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RESEARCH

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In vivo studies on evaluation of endophytic fungi extract from *Trichoderma viride* against cervical cancer

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Abstract

Background The crude ethyl acetate *Trichoderma viride* extract obtained from *Ziziphus mauritiana* was initially analyzed by HPLC for identification of major bioactive compounds, and then, it was subjected for in vivo acute and sub-acute toxicity, cervical cancer studies using Wistar albino rats.

Result During acute toxicity studies, animal groups treated with distinct dosage of 2000 mg/kg restrained toxicity signs in tested groups compared to controls for 14 days which established to be secure and non-toxic even at high dose. However, in terms of sub-acute toxicity studies, animals were given with repeated amount of (10 mg/kg, 20 mg/kg and 40 mg/kg) for a period of 28 days along with control group. Upon investigations of hematological, biochemical and histopathological studies repeated dose of 20 mg/kg and 40 mg/kg of *T. viride* extract found to be normal and no other major changes observed among treated groups. During in vivo studies, after treatment of *T. viride* extract (40 mg/kg) effectively inhibited the cervical cancer cell growth in DES-treated groups. Through HPLC analysis the major compound ursolic acid and 2,5-piperazinedione were mainly identified.

Conclusion The secondary metabolites of endophytes have been used substantially for the sustainable production of therapeutically important compounds. The limited availability of bioactive principles in plant sources could be surpassed by exploiting the chemical entities in the endophytes. In the present investigation, it has been accomplished that ethyl acetate extract of *T. viride* was safe at higher and lower dosage could be considered for pharmacological studies from plant may provide an excellent avenue for the discovery of drug candidates against deadly cancer diseases.

Keywords *Ziziphus mauritiana*, *Trichoderma viride*, Ursolic acid, Acute toxicity, Anticancer

1 Background

Fungus is considered as second largest kingdom on earth found on the surface of rock, mountains and sea water also in tropical, temperate and Antarctic ice regions [1, 2]. They have numerous natural products with diverse therapeutic properties. Among fungal community, endophytic fungi generates a wide range of secondary metabolites by producing higher level of enzymes, antibiotics, polysaccharides, organic acids, vitamin, antioxidants, pigments and pest control agents [3, 4]. Through signaling molecules the bioactive compounds for instance alkaloids, flavonoids, phenols, terpenoids were turned out commencing from roots of an individual plant through which fungi was attracted from the rhizosphere toward colonizing in the interior host plant as endophytes [5]. Many researchers were investigated several endophytic fungi from different therapeutic plants, marine source even in soil and so on [6]. Also there are some studies that have been conducted for the production of bioactive

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compounds, biodiversity, reproduction, biotransformation, taxonomy study and plant physiology in endophytic fungi [7, 8]. Furthermore, investigations indicated endophytes are not host specific, but it could envisage a range of similar host parts like stem, leaves, roots, twigs and could be isolated from different families and classes belonging to different plants grown under geographical and ecological conditions [9]. It has been hypothesized that plants from ethno-botanical history could be good candidates for endophytic fungi, providing unique bioactive compounds.

Among *Trichoderma* spp (TRS), the strain of *Trichoderma viride* produces several lytic enzymes and antibiotics. It is most widely distributed in the top soil habitats also used as biocontroller of soil-borne pathogens in plants [10]. Recently the study suggested that alcoholic extract of *Trichoderma viride* contains triterpenoids and exhibits potent antibacterial and antifungal activities. In our previous investigations, phytochemical analysis was performed using ethyl acetate extract of *T. viride* obtained from *Ziziphus mauritiana* which revealed the presence of secondary metabolites, namely terpenoids, tannins, anthraquinones and coumarins, respectively [11]. Furthermore through HPLC analysis six compounds have been identified in which ursolic acid, pentacyclic triterpenoids have been given prominent importance since it has numerous pharmacological activities such as anticancer, antimicrobial, hepatoprotective, antimalarial, antidiabetic and so on. In the present study, *T. viride* fungal crude extract was further analyzed for the presence of any toxic substances by acute and sub-acute toxicity and anticancer effects that offer human health benefits.

2 Methods

2.1 Cultivation and extraction

A *Trichoderma viride* (TRV) was isolated from the stem of *Ziziphus mauritiana* maintained in the medicinal plant garden of Mohamed Sathak College of Arts and Science (MSCAS) with the laboratory record number (TRV362) and authenticated by Dr. R. Jayaraman, Director, Plant Anatomy Research Centre, Chennai, India. The pure mycelial agar plugs were excised from the seven-day-old fungal culture measuring with thickness of 4 mm and 10 mm in diameter introduced into Erlenmeyer flask (500 mL) consisting 200 mL of sterile potato dextrose broth (PDB) medium and kept at 28 °C and 90% of RH for 21-day period. The flasks were completely monitored periodically for any microbial contamination [12, 13]. After incubation period, mycelial part was removed using sterile Whatman No. 1 filter paper and then obtained filtrate was preserved for further extraction process. An equal volume of filtrate and solvent ethyl acetate is taken in separating funnel and continuously shaken for 15 min until the formation of two immiscible layers. The upper portion of solvent was collected using separating funnel and kept for evaporation in fume hood, and the lower portion containing medium and other particles was discarded. Following that the obtained dried crude extract was used for further studies.

2.2 HPLC analysis

HPLC-graded methanol (1 mL) was subjected to (1 mL) of fungal crude extract and sonicated for 10 min. The sample along with standard centrifuged at 3000 rpm for 15 min, made up to 10 mL (not for standard) with methanol followed by filtration using 0.22-µm filter. Further 20 µl of standard was infused for the progression of ursolic acid chromatogram for 10 min. A dual gradient with movable phase contains (Solvent-A) 35% Milli-Q water and (Solvent-B) 45% methanol. Flow rate is constantly kept at 1 mL/min along with solvent gradient elution (0–10 min 55% A and 45% B) [14].

2.3 Experiment animals

Healthy adult young female Wistar albino rats were chosen for toxicity studies, weighing 150–200 gm, aged (6–8 weeks old) weeks obtained from Biogen, Bangalore. The experiments were intended and conducted at Animal House Facility of C.L. Baid Metha College of Pharmacy, Department of Pharmacology, Thoraipakkam, Chennai-600 097, Tamilnadu, India. The experimental research proposals were authorized by IAEC submitted to CPCSEA, Ministry of Environment and Forests, New Delhi (No: 12/321/PO/Re/S/01/CPSEA dated 11/10/2019), and carried out through recent guidelines for the concern of laboratory animals. The study animals were acclimatization toward laboratory atmosphere for 7 days before conducting the experiment. The obtained rats were maintained in room temperature at (22 °C ± 3 °C) under natural light/dark cycle, with relative humidity of at least 30% and not exceeding 70% other than throughout room clean-up; the aim should be 50–60%. During this process, rats were housed in cages, Hamster pellet (purchased from Adpx Pharma Private Ltd, Bengaluru, India) and clean water was provided. Illumination must be simulated; 12-h light/dark progressions beside with laboratory conventional food by unconstrained distribution of drinking water were provided [15].

2.4 Acute toxicity studies

Acute oral toxicity was firm by using three female Wistar albino rats divided into two groups, and it was marked in the tail. The tested animals were destitute of food (ad libitum) during the night (except water 16–18 h) previous to direction of the endophytic fungal extract. An ethyl acetate crude extract of *T. viride* mg/kg was suspended

in Tween 20, and it was administered to tested groups as single oral dose along with control groups. Following administration, ad libitum was suspended additionally 3–4 h. Each animal was cautiously monitored and recorded by measuring their weight, consumption of food, water, behavioral changes, skin changes, aggressively, mobility, feeling to noise and pain, respiratory activities and mortality. After 24 h, the number of survivors was noted and monitored for 14 days, later animals were forfeit and exposed to food, water consumption, body changes, and body weight was completely studied [15, 16].

2.5 Sub-acute toxicity studies

As stated by OECD guidelines-407, recurring dosage of oral toxicity study was performed in rodents intended for 28 days. Briefly, eighty young adult albino rats (40 males and 40 females) were selected with body weight of 150–220 gm and furthermore divided into four groups. Nearly four groups were treated in which initial group treated as control; further, 3 groups were subjected with ethyl acetate extract from endophytic fungi TRV at a low to higher dose (10 mg/kg, 20 mg/kg and 40 mg/kg body weight) premeditated as of acute oral toxicity of dose (2000 mg/kg), respectively. During treatment, rats were feed among regular food and water. Following administration of drugs, the tested animals were observed on every day for any changes of clinical signs, mortality until completion of experiment and body weight was documented on day 1, 7, 14 and 28 biweekly during study period. In the same way food and water consumption was calculated including tested and control groups. On 28th day, animals were sedated with isoflurane, blood samples were collected in potassium EDTA for hematology, sodium heparin for biochemical analysis along without anti-coagulant sample, and it was centrifuged at 3000 rpm for 10 min, respectively [16, 17].

2.6 Animal study

The effect of TRV extract on cervical cancer-induced animals was investigated in which four groups of Wistar albino female rats consist of 6 rats in everyset. Among them group I serves up as control, whereas carbon tetrachloride (CCl_4) was persuaded in groups II, III (20 mg/kg), IV (40 mg/kg) received (DES 0.5 mg/kg) once in a week intra-peritoneally for eight repeated weeks, following that thirty minutes each rats were injected with cortisol at 20 mg/kg body weight intra-peritoneally. Before sacrificing, each animal's body weight was measured and further killed by cervical decapitation. Following that blood was collected from experimental rats and serum was separated using centrifuge (1000×*g* for 10 min at 4 °C) for biochemical analysis; using 0.1 M phosphate buffer saline (PBS 1:9) liver tissue was washed twofold subsequently blotted and desiccated. For histological examination, cervical tissue was fixed in formalin [18].

2.7 Laboratory investigation

The collected samples were subjected for hematological and biochemical analysis. The anticoagulated EDTA whole blood was examined for complete blood count (CBC) through automated Mindray cell counter. Secondly, serum blood sample was estranged specific parameters like total protein, albumin, creatinine, potassium, urea, sodium, liver parameters, CA125, antioxidant assays such as lipid peroxidation, catalase, superoxide dismutase and tumor markers alike TNF-α assay, estradiol were analyzed Accurex diagnostics.

Furthermore histopathological examination was studied in which the dissected organs were immediately transferred into 10% buffered formalin and preserved for 24 h, followed by tissue preparation like dehydration, clearing, infiltration of wax, embedding and staining with hematoxylin–eosin for microscopic observation. Likewise the same procedure was followed for cervical tissue tumor analysis.

2.8 Statistical analysis

The statistical investigation was conceded by software GraphPad version 8.1. The obtained results were expressed as mean ± S.E.M. and accessed via one-way ANOVA Dunnett's comparison analysis from each group along with control for acute and sub-acute toxicity studies. The significance point was rest at 5% ($p < 0.05$); further, data were considered statistically.

3 Results

3.1 HPLC analysis

HPLC chromatogram of *T. viride* fungal crude extract showed various peaks at 200 nm wavelength; on the basis of retention time, 6 compounds have been identified. The highest peak was preferably considered for the presence of active chemical compound. Therefore by calculating the area of standard and test concentrations, it has been clearly depicted that *T. viride* sample consisting of 265.123 µg/mL concentration of ursolic acid compound compared to the standard and the concentration of test was calculated using the below formula, where C1 is concentration of standard, C2—concentration of test, A1—area of standard, A2—area of test. However, from this result it has been confirmed that *T. viride* crude extract contains increased amount of pentacyclic triterpenoids ursolic acid compared to other metabolites and RT values are given in Table 1 and the chromatogram is revealed in Fig. 1.

Table 1 HPLC chromatogram of *Trichoderma viride* extract at 200 nm

Peak#	Retention time	Area	Expected compound
1	1.787	39,822	Unknown
2	2.247	401,397	Unknown
3	2.460	289,579	2,5-Piperazinedione
4	2.687	62,148	Unknown
5	2.880	10,037	Unknown
6	3.313	40,759,291	3-Beta-3-hydroxy urs-12-en-28-oic acid (or) Ursolic acid

3.2 Acute toxicity study

After administration of ethyl acetate crude extract *T. viride* dosage at 2000 mg/kg does not have any toxicity signs, mortality, changes in behavior among tested groups contrast to the control group throughout 14 days of regular observation throughout experimental period. The body weight of the fungal extract-treated albino rats was detected 270.4 ± 21.24 on 1st day, 271 ± 3.64 on 7th day and 271.4 ± 2 on 14th day, respectively. There was no significant difference seen on above days statistically. Similarly, the water intake of *T. viride* extract-treated groups was observed on 1st day 58.2 ± 1.1, 58 ± 1.14 on 7th day and 59.20 ± 24 on 14th day along with the controls. Likewise, food intake exposed to fungal TRV-treated groups showed 59.6 ± 1.63 on 1st day, 59.6 ± 2.62 on 7th and 60.1 ± 5.38 on 14th day. Hence these results suggested that higher oral dosage of fungal extract confirmed no significant transience of the tested rats, indicating that yet high margin of drug was safety (Figs. 2, 3, 4). This study provided median lethal dose (LD_{50}) estimation representing the dosage which eradicates around 50% of the skilled populace. Therefore, it was concluded that *T. viride* extract was found to be non-toxic and LD_{50} value considered as 2000 mg/kg body for oral toxicity.

3.3 Sub-acute toxicity studies

During sub-acute toxicity studies (10 mg/kg, 20 mg/kg and 40 mg/kg) 3 various dosage of fungal crude extract was given to the animals. The repeated dose of fungal extract was liberated of exhilarating signs in animals all over 28 days. Subsequent to frequent quantity of *T. viride* extract-treated albino rats was detected for 28 days, 40 mg/kg; the body weight animals found to be 283.45 ± 3.25 on 28th day, 292.25 ± 2.34 observed in 20 mg/kg and 262.22 ± 3.54 in 10 mg/kg. Throughout this study, the percentage of albino rats body weight gets increased at 40 mg/kg/day from day 0 to 28 compared to controls, whereas at 10 mg/kg and 20 mg/kg treated groups, sustainable increase of body weight was observed commencing day 14 till day 28. The obtained results were evidently depicted (Fig. 5). However in case water and food intake was increased rate from day 0 to 28th day simultaneously. This indicates that all the experimental animals have taken feed and food in normal amount as their requirement (Fig. 6, 7). Similarly there were no physical changes, mortality and abnormal deviations initiated duration the entire research. In the same way, there was no significant change noted among laboratory investigations comparatively to control groups which clearly represent to facilitate *T. viride* extract was not lethal toward the body organs like spleen, liver, kidney.

3.4 Effect of *T. viride* extract on hematological parameters

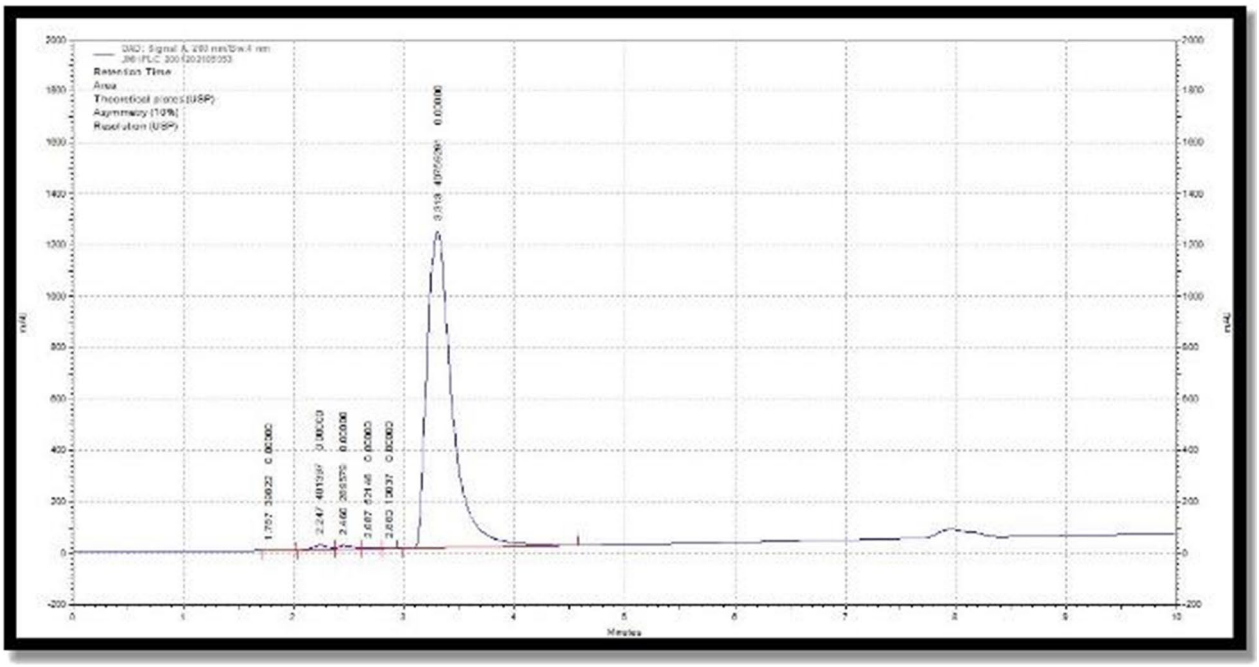
The experimentally observed values for hemoglobin for 10, 20 and 40 mg/kg were 14.4 ± 0.06, 14.6 ± 0.12 and 14.6 ± 0.18 g/dL for red blood cells obtained values that were 5.2 ± 0.86, 5.2 ± 1.2 and 5.2 ± 0.92 (10^6/mm^3) correspondingly. In the same way, white blood cells in low groups found to be 8.421 ± 1.10, mid-groups 8.422 ± 2.63 and 8.423 ± 1.06 in high dose-treated groups ($\times 10^3$/mm^3), whereas packed cell volume among three set was 48.2 ± 0.31, 48.3 ± 0.62 and 48.8 ± 1.26%, respectively. The basic hematological parameters between three experimental groups 10, 20 and 40 mg were similar as compared to control groups (Fig. 8).

3.5 Effect of *T. viride* extract on biochemical studies

The biochemical examination was conceded to ensure about toxic effects of fungal crude extract of *T. viride* when administered orally for period of 28 days. Through blood parameters each functions of organs such as liver, spleen and kidney could be easily identified. Upon investigations, there were no significant changes observed in creatinine, total bilirubin, SGPT and SGOT by sub-acute toxicity analysis. However, there was increase in alkaline phosphatase observed in 40 mg/kg compared to control. Similarly, the value of urea found to be statistically insignificant to 20 mg/kg as compared to other groups. Instead, there was increase in total protein, albumin observed in low dose 10 mg/kg found to be statistically insignificant as compared to control, whereas 20 mg/kg (mid-dose) and 40 mg/kg (high dose) were found to be statistically significant. Likewise, the values of potassium to 20 mg/kg were decreased, but 40 mg/kg found as normal contrast toward control. From Fig. 9 it was clearly depicted that statistical interpretation of various biochemical parameters between 20 mg/kg and 40 mg/

a). A representative HPLC chromatogram of JM

[PDA Multi 1/200 nm/Bw: 4 nm]



b). A representative HPLC chromatogram of standard

[PDA Multi 1/200 nm/Bw: 4 nm]

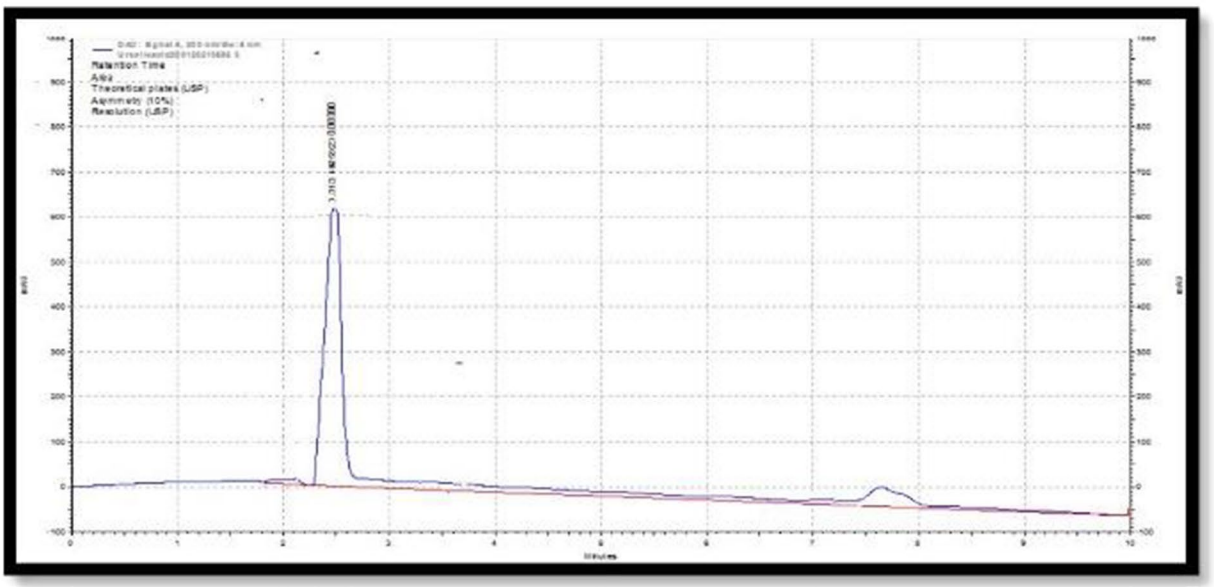


Fig. 1 **a** A representative HPLC chromatogram of JM [PDA Multi 1/200 nm/Bw: 4 nm]. **b** A representative HPLC chromatogram of standard [PDA Multi 1/200 nm/Bw: 4 nm]

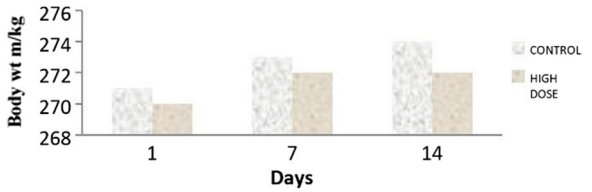


Fig. 2 Change in body weight (mg/kg) of the animals exposed to TRV

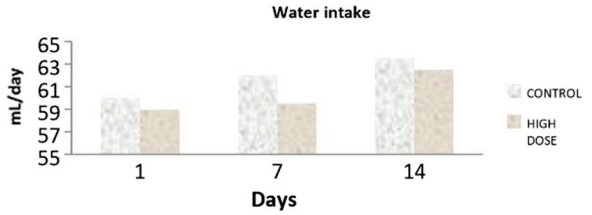


Fig. 3 Change in water intake (mL/day) of the animals exposed to TRV

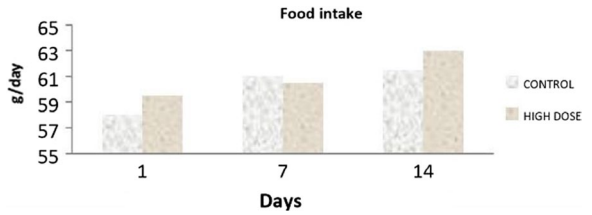


Fig. 4 Change in food (g/day) of the animals exposed to TRV

kg found to be non-considerable as compared to control, whereas low dose-treated groups are comparatively higher than control and other treated groups.

3.6 Assessment of histology

Subsequent to repeated administration of *T. viride* crude extract for 28 days, histopathological analysis was investigated. On comparison with the control group drug liver cells are found to have normal portal tract, hepatic artery, bile ducts and dilation of central hepatic veins observed in three groups of TRV extract. Histological spleen showed normal mesangial cells, central arteriole concentrated on treated groups, while histological examination of kidney revealed normal glomeruli, medulla and cortex in 20 mg/kg and 40 mg/kg dosage. Lymphocytes present within the areas where no capillaries can be seen in drug-treated groups, venous sinus slightly inflamed, venous congestion and neutrophils are prominently seen. Based upon these studies it was clearly depicted that liver, spleen and kidney are being normal even after constant administration of fungal extract from low to high doses (Fig. 10).

3.7 In vivo Anticancer studies

3.7.1 Determination of body weight and ovary weight

After administration of selected dose each group of animals was observed for its body weight and ovary weight. On observing, it has been found that slight increased and decreased body weight of the animals from positive control (DES), *T. viride* fungal crude 20 mg/kg and 40 mg/kg administered groups as contrast toward control group. The initial body weight for Group II (DES + CCL_4) at 0.5 mg/kg dose was 245.04 ± 1.10, and final body weight was found to be 281.11 ± 1.10. In Group III, TRV fungal crude 20 mg/kg showed 239.12 ± 1.15 at initial stage; however, body weight gets reduced to 140.32 ± 1.34 at final stage. Similarly in Group III, TRV fungal crude 20 mg/kg treated groups showed body weight of 242.14 ± 1.42 at initial and changes in body weight were 135.18 ± 1.62 observed finally, while in control group body weight observed at initial was 250.14 ± 2.21 and increased up to 281.11 ± 1.10 as final body weight of the animal groups (Fig. 11). The ovary weight observed in Group I was 94 ± 0.22 with relative ratio of 2.65 ± 0.11. In Group II weight of the ovary was 138 ± 0.32 with relative ovary/body weight ratio found to be 1.78 ± 1.21. Group

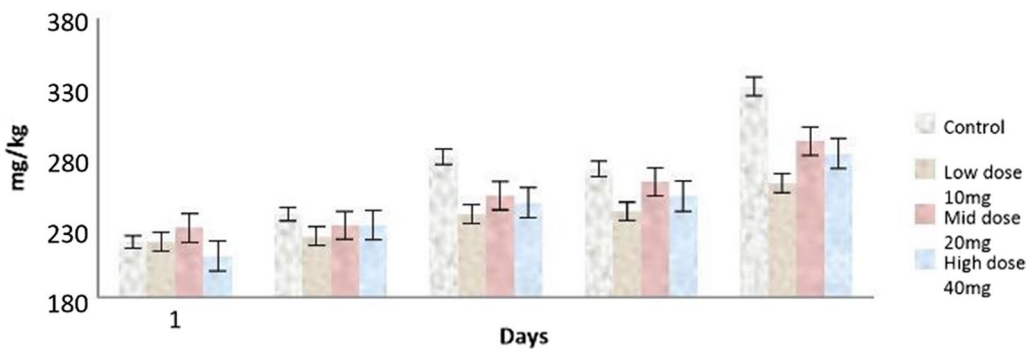


Fig. 5 Changes of Wistar albino rat's body weight exposed to TRV

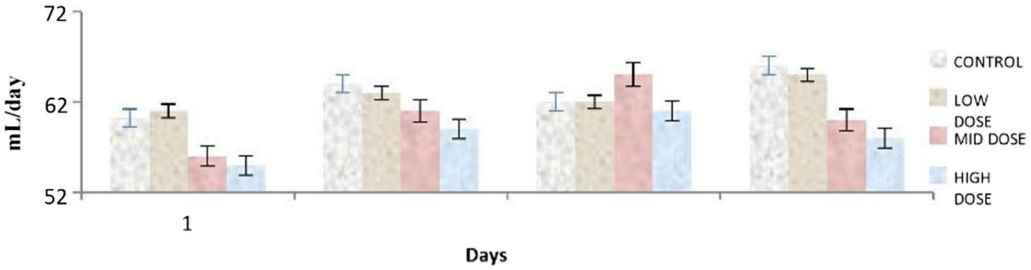


Fig. 6 Changes of Wistar albino rat's water intake (mL/day) exposed to TRV

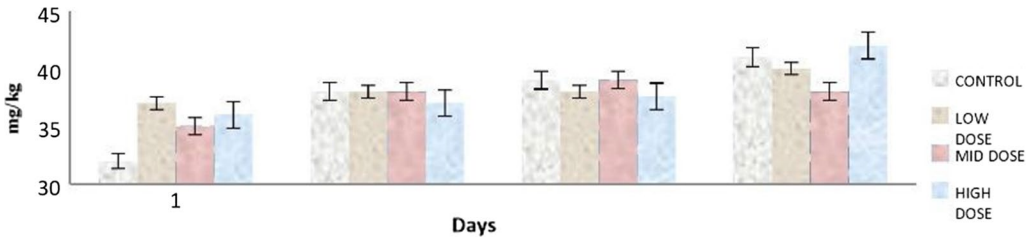


Fig. 7 Changes of Wistar albino rat's food intake (g/day) exposed to TRV. NS—not significant, **($p > 0.01$), *($p > 0.05$), $n = 10$ values are mean ± S.D (one-way ANOVA followed by Dunnett's test). *Note Trichoderma viride* fungal crude extract

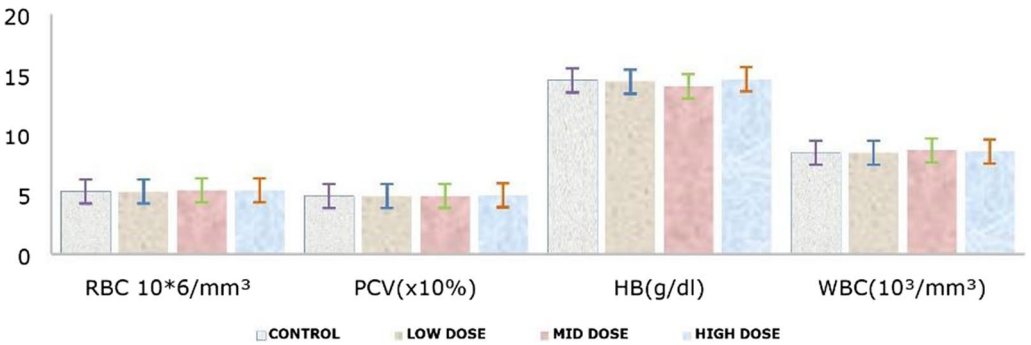


Fig. 8 Hematological observation of *T. viride* crude extract

III-treated groups found to have 111 ± 0.46 ovary weight, and its relative ratio was 2.16 ± 1.51. However in Group IV-treated animal groups ovary weight was 101. ± 0.20 observed with relative ovary/body weight ratio as 2.40 ± 1.02, respectively.

3.8 Tumor marker analysis

The crude extract of TRV possesses anticancer activity against cervical cancer. The extract at two different concentrations 20 mg/kg and 40 mg/kg was checked against cancer antigen (CA-125) tumor marker of cervical tissues along with the control as described in Fig. 12. The obtained results suggested that the values for CA-125 found to be 24.52 ± 1.17 at 40 mg crude extract of TRV, whereas at 20 mg crude extract of *T. viride* showed 67.85 ± 1.21 correspondingly. However in control group it was found as 12.38 ± 1.05 and 35.81 ± 1.27 noted in DES-treated groups, respectively.

3.9 Oxidative stress enzyme

Superoxide dismutase (SOD) is a key oxidative stress enzyme which has the ability of scavenging extensive range of free radicals. The SOD observed in Group III was 6.11 ± 0.42; in Group IV 7.59 ± 0.06 was observed, respectively. But in control group it was found to be 8.48 ± 0.22; similarly, in Group II the SOD was 5.62 ± 0.35 in the treated groups. Further catalase enzyme was studied in which treated groups had catalase enzyme level ranged from 1.94 ± 0.23, 2.20 ± 0.21 and 2.83 ± 0.18 for DES-treated Group II, followed by Group II 20 mg crude extract of *T. viride* and Group III 40 mg crude extract, respectively. But in control treated groups catalase was

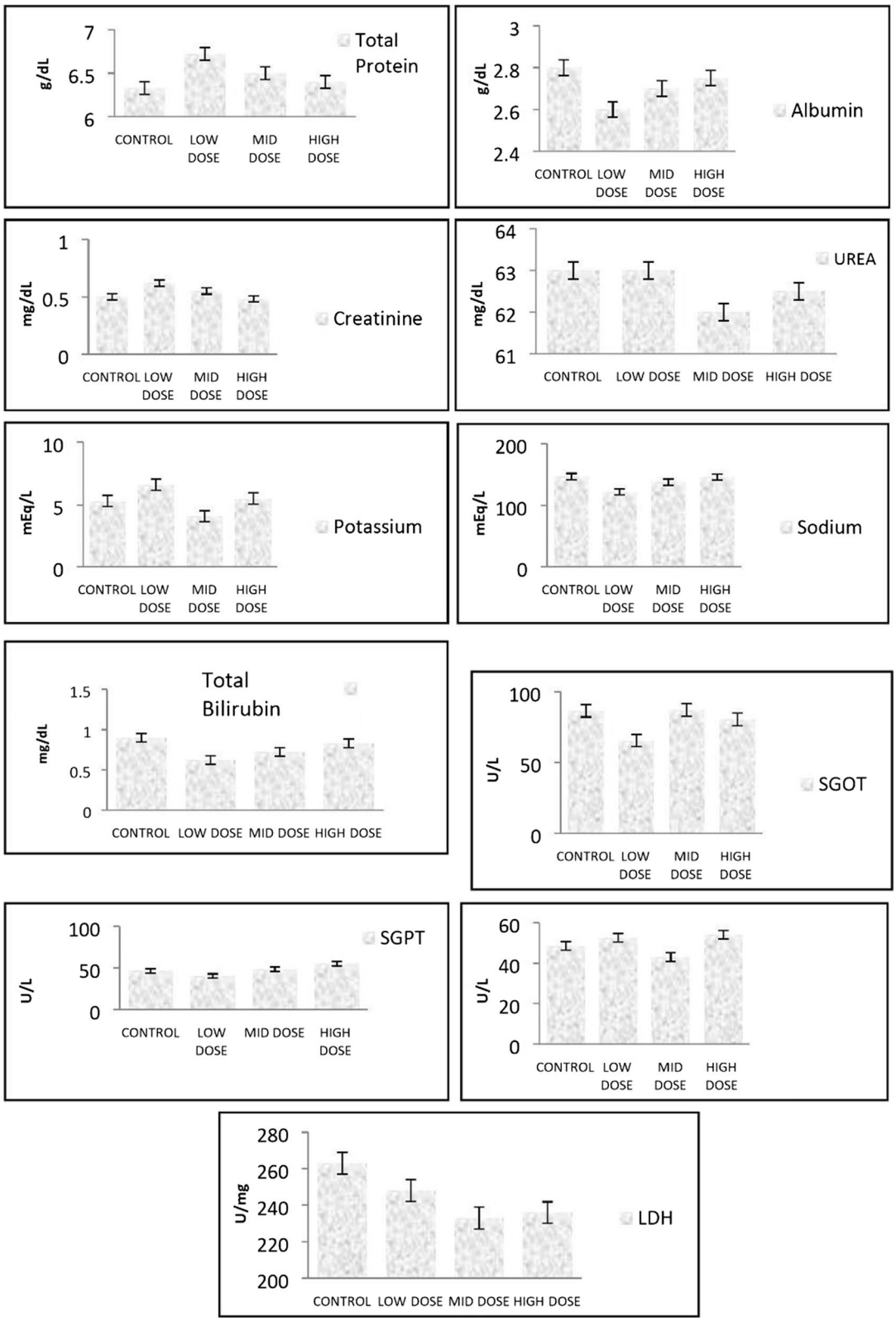


Fig. 9 Effect of *T. viride* on biochemical kidney/liver parameters

found to be 3.18 ± 0.16. Thirdly lipid peroxidation was carried out; among the treated groups, lipid peroxidation was observed in which 4.04 ± 1.32 observed in Group II-treated groups. In 20 mg of crude extract *T. viride*-treated groups lipid peroxidation enzyme was 2.98 ± 1.22 and 1.96 ± 2.42 observed in 40 mg crude extract-treated groups, but in control 1.67 ± 2.31 was observed (Fig. 13). This showed there is a significant variation pragmatic among the control and treated groups with the p value ($p < 0.001$).

3.9.1 Biochemical parameters

After intraperitoneal administration of TRV extract, biochemical parameters were commonly studied. The liver marker enzyme aspartate aminotransferase (AST/SGOT) prominent marker enzyme was analyzed; among the treated groups, an increase of AST observed in Group II treated with DES was 480.15 ± 1.31, Group III-treated 20 mg crude extract of *T. viride* was found 375.04 ± 1.16; however, in Group IV 40 mg crude extract of *T. viride* was 223.14 ± 1.21 and control group ranged 120.22 ± 1.22. Likewise results obtained from alanine aminotransferase (ALT/SGPT) were found to be higher in Group II 140.12 ± 1.11; least for 40 mg crude extract of *T. viride* was 80.13 ± 1.03 followed by 20 mg crude extract of *T. viride* found to be 102.21 ± 2.02 and the normal control 69.11 ± 2.03, respectively. However, there was an increase value of alkaline phosphatase (ALP) observed in Group II ranged 583.08 ± 1.48. Oher groups ranged between 493.16 ± 2.16, 341.32 ± 2.09 and 251.10 ± 1.12 for 20 mg crude extract of *T. viride* Group III, 40 mg crude extract Group IV and control group, respectively. However with all this results clearly indicated that the control and treated groups were statistically significant with DES-treated groups alone with the p value ($p < 0.001$) by ANOVA pursued by means of Dunnett's "t" test. The total protein for DES-treated group was 116.02 ± 1.16. However in 20 mg of crude extract-treated groups, total protein was 94.13 ± 2.32 and in Group IV 40 mg T. *viride* crude extract has 64.14 ± 2.07 and for the control group 56.18 ± 1.08 total protein was observed. The results obtained from 40 mg T. *viride* crude extract were 1.02 ± 1.20, followed by 1.14 ± 1.18 in 20 mg T. *viride* crude extract, whereas in DES-treated animals have increased amount of total bilirubin 2.13 ± 1.15, respectively. The control has least amount of bilirubin 0.81 ± 2.21 value. From the experimental group albumin was lower in Group II treated animals 1.96 ± 1.12. The *T. viride* crude extract 20 mg treated groups have 4.10 ± 1.11 and 40 mg treated groups found to be 5.53 ± 1.04; however, control groups contain 6.28 ± 1.21 of albumin level.

Furthermore, the pro-inflammatory marker TNF-α and estradiol were studied in which the results obtained from TNF-α present in the control group were 578.21 ± 2.12. Although TNF-α found in 40 mg T. *viride* crude extract was 687.10 ± 1.12 followed by 20 mg of crude extract 877.11 ± 2.10. However in DES-treated Group II increased level of TNF-α was 1734.04, respectively. The estradiol was found to be 96.8 ± 7.4 in DES-treated groups, in the same way 72.1 ± 1.21 was observed in 20 mg crude extract *T. viride*, and in 40 mg 54.6 ± 1.51 of estradiol was present as described in Fig. 14. The control group has least amount of estradiol 30.4 ± 1.08 value, respectively.

3.9.2 Evaluation of cervix tissues by histopathology

The histopathology of the four set of animals resembling Group I—Control, Group II—DES + $CCL_{4,}$ Group III—20 mg T. *viride* crude extract and Group IV—40 mg T. *viride* crude extract showed varied histology in cervical tissues. However, there was a neoplastic squamous cell and chronic inflamed stroma was observed in Group II, whereas in Group III and IV drug-treated *T. viride* crude extract of 20 mg and 40 mg showed reduced size in squamous cells and also reduction in inflammation of stroma. At the same time as in Group I control treated animals showed normal stroma and squamous cells, respectively (Fig. 15).

4 Discussion

Secondary metabolites from endophytic fungi naturally have high efficiency of therapeutic values for treating various kinds of diseases. Most of the endophytic fungi utilize plant source by producing same bioactive compounds through biotransformation process [19]. Likewise, in the present study HPLC results denoted the presence of bioactive compound, namely 3-beta-hydroxy urs-12-en-28-oic acid from *Trichoderma viride*. Some researchers reported microbial transformation of 3 polar metabolites 3β, 16α-dihydroxy-olean-12-en-28-oic acid, 3-oxo-16α-hydroxy-olean-12-en-28-oic acid and 28-*O*-β-D-glucopyranoside from the *Nocardia corallina* endophytes [20]. Naturally pentacyclic triterpenoid majorly found in several plant species such as *Prunella vulgaris, Gardenia jasminoides Ellis, Crataegus pinnatifida Bunge* and *Ziziphus mauritiana* [21, 22]. Likewise, tritepene compound echinocystic acid was obtained from *Echinocystis fabacea* which has substantial inhibition of cytotoxic effect against various *in vitro* cell line HEK-293, HeLa, L929, WEHI-164 and HEpG2. Abdallah et al., 2016 synthesized bioreduction of silver nanoparticles from *T. viride*; it was spectrophotometrically observed and characterized [23].

Based upon HPLC analysis, the crude extract of *T. viride* was further analyzed for acute oral toxicity study. Into this, a particular dosage of crude extract 2000 mg/kg was directed orally on rats intended for 14 days to check any behavioral changes, body weight, food and water consumption, etc. These results suggested that higher oral dose 2000 mg/kg TRV extract proved no considerable mortality of tested rats, indicating that even high margin of drug was safety. This study provided an estimation of median lethal dose (LD_{50}) of *T. viride* crude extract was found to be non-toxic and LD_{50} value considered as 2000 mg/kg exhibited with lower toxicity. Similarly study conducted in Sprague Dawley rats with median lethal dose of *T. viride* chitinase found that distinct dosage of 2000 mg/kg showed no evident symptoms of animal death [15, 18]. Medhat et al., isolated *Trichoderma viride* from mangrove soil in Egypt; they have optimized chitinase activity using submerged fermentation. The purified chitinase showed effective antifungal activity adjacent to *Fusarium oxysporum*. Further, in vivo studies carried out using chitinase with median lethal dose of Sprague Dawley rats an average body weight (18.43) mg/kg. In the lead of MTT assay, purified chitinase has noxious end product with an IC_{50} value 20 µg/mL to MCF7 and simultaneously in HCT-116 cell lines IC_{50} value 44 µg/mL was observed [20].

Subsequent exposure to certain toxic substances leads to change in the body weight, and other internal organs would reflect toxicity, and moreover, if their loss of body weight more than 10% it will be considered as statistically considerable with adverse effects of drug [24, 25]. Therefore in the current observation, animals body weight in the treated groups was not significant unlike among themselves, so it was concluded that *T. viride* fugal extract is almost non-toxic and secure for exploit. An additional aspect was studied from acute toxicity of water and food intake for a period 14 days; hence, they were not influenced by administration of *T. viride* fungal extract; as a result, it did not proceed any appetite and precarious impacts. In case of sub-acute toxicity studies three different doses as 10 mg/kg, 20 mg/kg and 40 mg/kg of *T. viride* crude extract were given to animals for 28 days; results indicated that the fungal extract was liberated of exhilarating signs in animals throughout the study phase. The percentage of albino rats body weight gets increased at 40 mg/kg/day from day 0 to 28 compared to controls, whereas at 10 mg/kg and 20 mg/kg administered groups, sustainable increase of body weight was experiential from 14th day to 28 days. Similar results have been obtained by (Kusakabe et al., 1980) which correlated our study [26]. After repeated dose of administration of the *T. viride* crude extract at 20 mg/kg, 40 mg/kg for 28 days, increased body weight was observed which is correlated with the study by [27] obtained same results of gaining body weight.

In terms of hematological, biochemical parameters absence of major changes was noted contrast toward control in the current investigations, as same results interrelated with the study [28]. However, immunological parameters neutrophils, eosinophils and platelets report noticeably increased ($^{**}p < 0.01$) comparatively to the control. Similar studies were experientially interrelated by various researchers obtained from different fungal extract [29]. Histological examination was done for assessing the treatment-related changes in cell structure of the organs and tissues [30]. As a result no significant abnormalities were observed in histopathological investigations of tissues and the organs, namely, liver, spleen and kidney were found to be normal. Similar results were attained by some of the researchers, in their treated animal groups [31–33]. Therefore, chronic dosage of the crude extract at sub-acute studies for 28 days showed no mortality and all the Wistar Albino rats showed normal histology, hematopoietic and biochemical parameters.

Further, the study was extended for anticancer activity in cervix using fungal crude extract *Trichoderma viride* after administration of DES (0.5 mg/kg) intraperitoneally to female rat carcinogenic effect was observed. The supplementation of female Wistar Albino rat with fungal crude extract 20 mg/kg and 40 mg/kg does not

(See figure on next page.)

Fig. 10 Histopathological examination of individual organs from TRV-treated groups. (a) Control. **A** Photomicrograph of liver in Wistar albino rats: Control group showed normal liver lobule architecture with central vein (v), normal hepatocytes, blood sinusoids and central vein. **B** Control group showed normal spleen with central artery, splenic cords (red pulp), lymphatic nodule (white pulp) and capsule. **C** Control group showed normal kidney with glomerulus (arrow), papillary ducts and capillaries. (b) Group I—low dose (10 mg/kg b.w.). **B** Photomicrograph of liver in Wistar albino rats: Low dose (10 mg/kg b.w) showed normal liver lobule architecture with central vein (v), normal hepatocytes, blood sinusoids and central vein. **B** Control group showed normal spleen with central artery, splenic cords (red pulp), lymphatic nodule (white pulp) and capsule. **C** Control group showed normal kidney with glomerulus (arrow), papillary ducts and capillaries. (c) Group II—mid-dose (20 mg/kg b.w.). **C** Photomicrograph of liver in Wistar albino rats: Mid-dose (20 mg/kg b.w) showed normal liver lobule architecture with central vein (v), normal hepatocytes, blood sinusoids and central vein. **B** Control group showed normal spleen with central artery, splenic cords (red pulp), lymphatic nodule (white pulp) and capsule. **C** Control group showed normal kidney with glomerulus (arrow), papillary ducts and capillaries. d) Group III—high dose (40 mg/kg b.w.). **D** Photomicrograph of liver in Wistar albino rats: High dose (40 mg/kg b.w) showed normal liver lobule architecture with central vein (v), normal hepatocytes, blood sinusoids and central vein. **B** Control group showed normal spleen with central artery, splenic cords (red pulp), lymphatic nodule (white pulp) and capsule. **C** Control group showed normal kidney with glomerulus (arrow), papillary ducts and capillaries

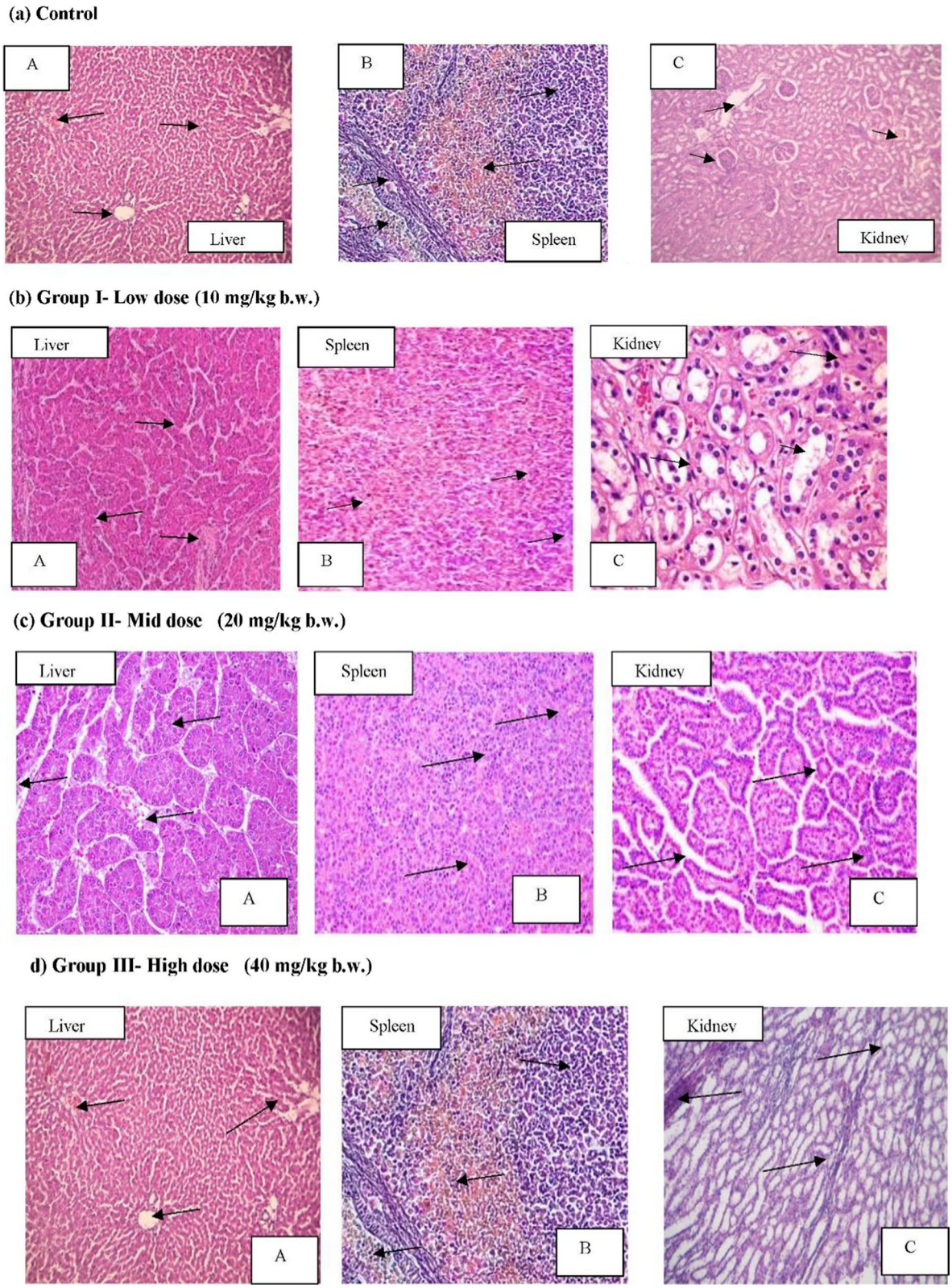


Fig. 10 (See legend on previous page.)

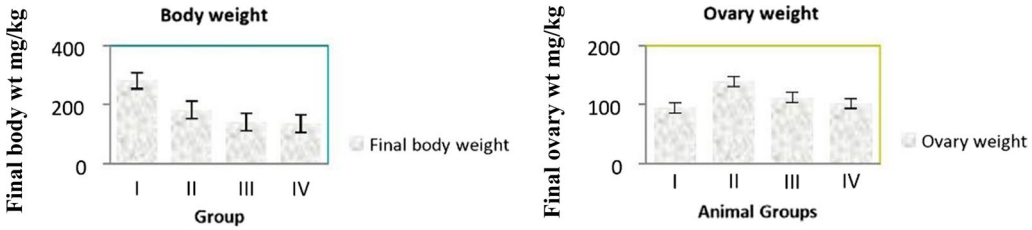


Fig. 11 Effect of *T. viride* fungal crude extract on body weight and ovary weight

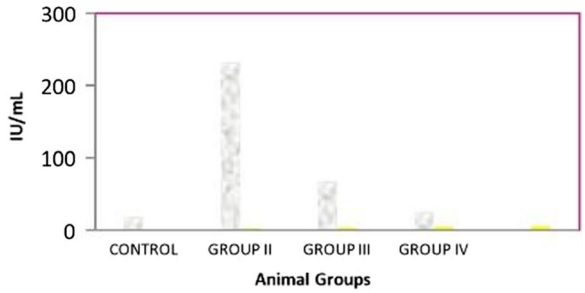


Fig. 12 Effect of *Trichoderma viride* extract on CA-125

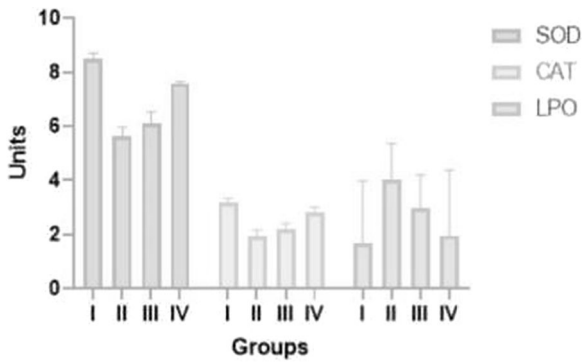


Fig. 13 Antioxidant levels of *Trichoderma viride* on different concentrations

induce several harmful effects such as mortality, body or ovary weight loss. Some studies have shown similar study as exactly associated with the existing result [34–36]. Through biochemical parameters, crude extract of 40 mg/kg relatively increased liver enzymes was observed, indicating as a sign of hepatoprotective properties. Comparable results were obtained by previous investigations which correlated our findings [37]. The results from the present study further demonstrated anti-inflammatory biomarker effect of *T. viride* crude extract on the secretion of macrophage-derived pro-inflammatory cytokines TNF-α and estradiol. As a result, *T. viride* crude proved an inhibitory end result on production of pro-inflammatory marker TNF-α and estradiol at 40 mg/kg compared to 20 mg/kg. Quinolactacin A1 and A2 were previously isolated from the genus *Penicillium* crude extract which also showed inhibitory activity against TNF-α involved in inflammation produced by macrophages which may lead to septic shock [38]. Additionally, cancer antigen (CA-125) tumor marker was tested among the groups in which decreased level of cancer antigen significantly reduced at 40 mg/kg crude extract of endophytic fungi; however, similar results were interconnected with the present study [39]. The antioxidant enzyme activity, namely SOD, catalase and lipid peroxidation, was further studied in our present study. All the three activities showed minimal significance ($^{**}p < 0.01$) in 40 mg/kg crude extract-treated group IV compared to other groups. However in the current exploration, SOD activity was stable above all phases within infected rats. Consistent with our results, there were an momentous changes of catalase activity observed throughout the experiment within infected animals, while lipid peroxidase activity was substantially reduced on 40 mg/kg crude extract-treated group IV compared to other groups. As the antioxidant activity was already reported, it can be proposed that decreases in total antioxidant activity which is correlated with our report [40]. Taking into account, present research showed significant reduction in the total antioxidant capacity that was detected in tainted rats and dissimilarity was identified among the infected and control rats [41]. Since, the current research was formerly demonstrated and reported to have efficient anticancer activity of *T. viride* crude extract in cervical cancer. Interestingly, crude extract showed evidence of major inhibitory effect on cervical metastasis. Treatment of cervical cancer induced in Wistar albino rats with 20 mg/kg and 40 mg/kg crude extract be evidence for reduction in cervical intraepithelial neoplasia (CIN) squamous cells size along with reduced inflammation of stroma upon histological examination. However in control, squamous and stroma cells are normal in size,

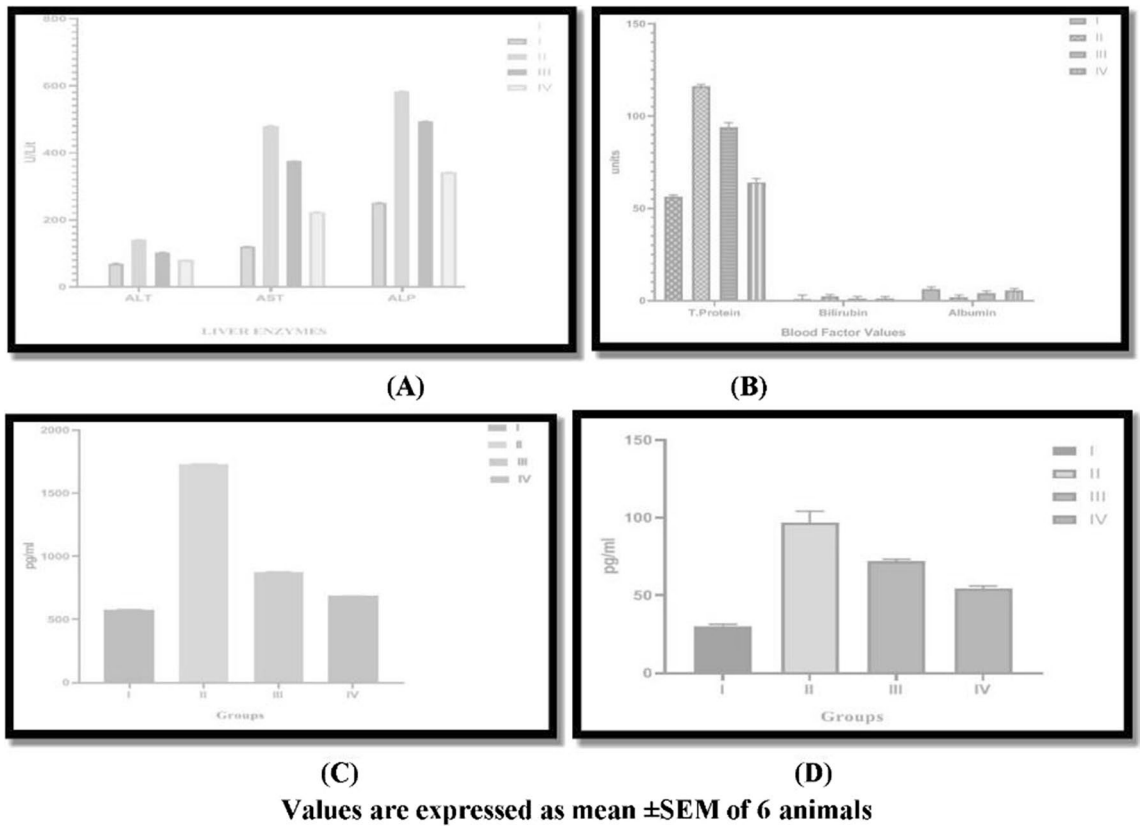


Fig. 14 Effect of *Trichoderma viride* crude extract on biochemical parameters. Group I (control) II (DES treated), Group III (*Trichoderma viride* extract 20 mg/kg) and IV (*Trichoderma viride* extract 40 mg/kg) ANOVA, followed by Dunnett's "*t*" test.** $p < 0.01$. ns represents non-significance. (**A**—liver enzymes, **B**—total protein, bilirubin and albumin, **C**—TNF-α and **D**—estradiol)

whereas DES + CCl4 showed neoplastic squamous cells and chronic inflamed stroma. These results indicate that DES was able to induce histological lesions prominently noted. Correspondingly, substantial evidence obtained from previous studies [42].

5 Conclusions

From these findings, HPLC analysis clearly indicated that *Trichoderma viride* fungal crude extract possesses pentacyclic triterpenoids, namely ursolic acid, which have a possible anticancer properties. Through acute toxicity studies, the crude extract of *Trichoderma viride* at concentration 2000 mg/kg body weight showed no toxic effects, which clearly indicated that this fungal extract is safe and does not produce any toxic effect. In view of the fact, no mortality observed in 14-day period which denotes that fungal extract has some medicinal properties. Similarly in sub-acute toxicity, when given repeated dose of mid- and high dose for 28 days proved to be safe and non-toxic to experimented animals. Upon biochemical, hematological and histopathological findings, TRV fungal crude is pertaining to sub-acute toxicity study. Hence this was the first attempt to study about the toxicity from TRV fungal crude obtained from the plant *Ziziphus mauritiana.* Based on the results it has been clearly identified that TRV crude can be utilized as effective anticancer activity in cervical cancer. This can be possible due to the intoxicating of bioactive compounds, namely pentacyclic triterpenoids ursolic acid. Studies on fungal diversity of endophytes have largely determined relationships between host plants and endophytic fungi for generating bioactive complex naturally. Therefore, commencing from this study it has been concluded that through biotransformation, the ursolic acid was transformed from *Ziziphus mauritiana* to *Trichoderma viride* and the fungal crude extract broadly used as major important component in pharmaceutical fields. Hence, *Trichoderma viride* found to be the most predominant among mycoflora genus shown to yield effective bioactive metabolites with cytotoxic, antimicrobial, antioxidant and anticancer activities.

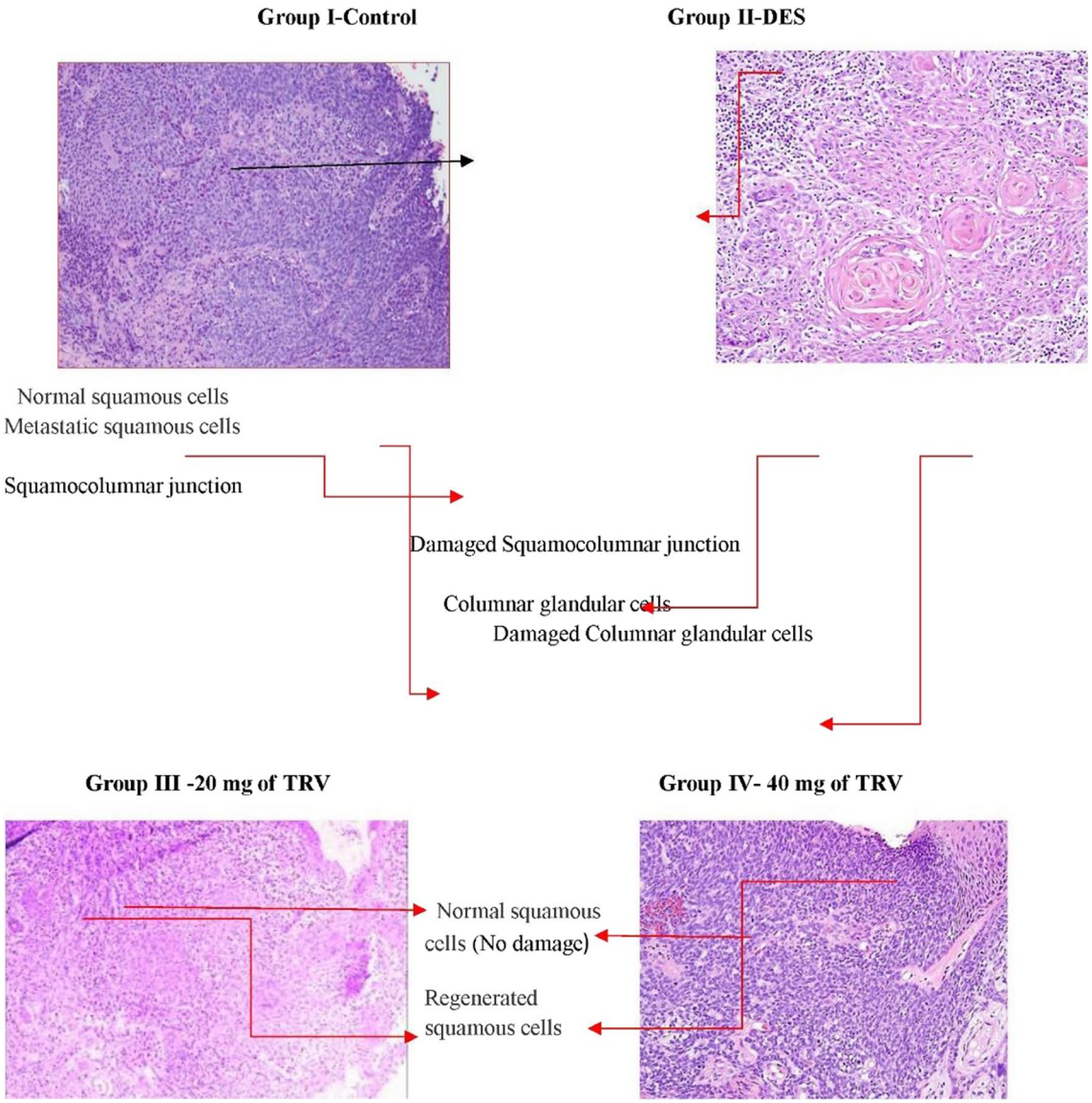


Fig. 15 Histopathological study of cervical cancer induced in Wistar albino rats. Histopathological analysis of cervical tissue. Nests of neoplastic squamous cells are regenerated (arrow head). The cancer is poorly differentiated and keratinizing (original magnification ×200). **B** Pelvic lymph nodes are no damaged in 20 and 40 mg of TRV. Columnar glandular cells and other surrounded by normal lymphocytes (original magnification ×200)

Abbreviations

%	Percentage
°C	Degree celsius
µl	Microliter
µm	Micrometer
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
CA125	Cancer antigen 125
CCL4	Carbon tetrachloride
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
DES	Diethylstilbestrol
DL	Deciliter
EDTA	Ethylenediamine tetraacetic acid
G	Gram
HCT	Human colon cancer cell line
HEK293	Human embryonic kidney 293
HEPG2	Human hepatoma cell line
HPLC	High-performance liquid chromatography
IAEC	Institutional Animal Ethics Committee
IC_{50}	Half-maximal inhibitory concentration
Kg	Kilogram
LD_{50}	Lethal dose 50
M	Molar
MCF7	Michigan Cancer Foundation-7
Mg	Milligram
Min	Minutes
mL	Milliliter
Mm^3	Cubic millimeter

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffer saline
PDB	Potato dextrose broth
RPM	Rotation per minute
SGOT	Serum glutamic-oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
TRV	*Trichoderma viride*
WEHI	Walter and Eliza Hall Institute of Medical Research

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Author contributions
SH and SMY conceived the study. VA,NY & GKS: Validation, SH: Performed the assays, collected the data and performed the data analysis. SH & SMY wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
The manuscript contains experiment animals, and it was performed in line with the principles of the Declaration of C.L. Baid Metha College of Pharmacy IAEC submitted to The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi. Approval was granted by the Ethics Committee of University B (11/10/2019…/ No: 12/321/PO/Re/S/01/CPSEA)."

Consent for publication
Not applicable.

Competing interests
All the authors are declared that they have no financial or non-financial conflict of interest.

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