**In vitro produced glycoalkaloids from Solanum nigrum L. and evaluation of their potential role as antibilharziasis**

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**Objective:** To evaluate the anti schistosomiasis activity of the bioactive formed glycoalkaloids from *Solanum nigrum* L. (family: Solanaceae) (*S. nigrum*) to control one of the most prevalent parasitic tropical diseases.

**Methods:** Murashige and Skoog media containing growth regulators were used for callus and regeneration establishment. High performance liquid chromatography analysis was used for identification and quantitation of the glycoalkaloids. Mice infected with cercariae were used for biological studies. Hepatic marker enzymes, urea cycle enzymes and antioxidant biomarkers as well as chromosomal aberrations of mice were measured before and after treatment. Histopathological examination for infected and treated mice was also carried out.

**Results:** High performance liquid chromatography analysis proved that *S. nigrum* cultures had the power to produce glycoalkaloids from calli and regenerate plants in higher concentrations than original plant. Treatment of infected mice with the separated glycoalkaloids induced significant improvement of all tested biomarkers. In addition, glycoalkaloids administration resulted in significant elaboration of somatic and germ cell mutation caused by bilharzia worms. Histopathological study illustrated improvement signs regarding inflammation and egg disintegrations. The refinement of biological signs was dose dependent.

**Conclusions:** The outcomes of this study indicated potential effect of *in vitro* cultures of *S. nigrum* for glycoalkaloids formation. The data proved the potent effects of the glycoalkaloids against the hazards of bilharzias' infection including liver, renal and chromosomal disorders. The data of the present study could be a tool for development of plant originated antibilharziasis medicine to dispose the danger of ultimate debilitating helminthes.

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1. **Introduction**

Bilharziasis is a neglected tropical disease which infects millions of world populations including Egyptians. Infection of *Schistosoma mansoni* (*S. mansoni*) is the causative agent of liver fibrosis of the host. Moreover, the disease induced hepatosplenomegaly, liver fibrosis and cirrhosis([1,2]). Schistosomiasis is the second neglected tropical disease among the most widespread parasitic diseases in sub-Saharan Africa. About 57 million poor health adjusted life-years are lost annually because of these diseases. In 2008, among 17.5 million of globally treated people for schistosomiasis, 66.86% (11.7 million) are from sub-Saharan Africa([3]). *Schistosoma haematobium* induced inflammation may enhance stem mutation([4]). Thus, bilharziasis infection exerts major health, social and economical burden to these countries. Treatment of bilharzias is complicated by the emergence of resistance worm strains to used drugs. These drugs are few and may have genotoxic hazards([5,6]).

Modern therapy turned to nature as a valuable source of bioactive materials. It is estimated that about 50% of all the drugs in newfangled medicaments are from plant origin([7]). Candidate plants for antibilharziasis may depend on ethnomedical informations.

*Solanum* plants (family: Solanaceae) were used traditionally as anti schistosomiasis agent. Water extract derived from leaves of *Solanum nigrum* L. (*S. nigrum*) had been utilized as a chemical to attenuate *S. mansoni* cercariae in mice([8]). *Solanum lycocarpum* (*S. lycocarpum*) with glycoalkaloids possessed an immunomodulatory effect on...
S. mansoni infected mice[9]. Solanum xanthocarpum ethanolic extract was effective against S. mansoni snail vector (Biomphalaria glabrata)[10]. Glycoalkaloid extract of S. lycoctonum was found to have defensive activity against mitomycin C stimulated mutation[11]. Glycoalkaloids are regarded as defensive allelochemicals against pathogens and predators as fungi, viruses, bacteria, insects and worms[12]. Due to defensive character, development of new cultivars of different Solanum species with high steroidal glycoalkaloid levels is going on. Besides, unstable glycoalkaloids content in S. nigrum even in the same day due to environmental and stress factors is also reported[12]. This instability represents a barrier for drug research from this natural important source.

The challenge of producing natural antibilharziasis drugs from plant origin lead to searching for alternative way rather than breeding for producing such valuable compounds. Different studies of in vitro glycoalkaloids production from Solanum species are reported[13-16].

The aim of the present work is to establish in vitro culture conditions suitable for glycoalkaloids bioformation from S. nigrum in constant uninterrupted intensity. An important destination of this study is to evaluate antibilharziasis activity of the formed bioactive glycoalkaloids through measuring hepatic marker enzymes, urea cycle enzymes as well as antioxidant biomarkers of infected mice before and after glycoalkaloids treatment. Amendment of bilharzias infected mice[9]. S. mansoni infected mice[9]. S. nigrum infected mice[17].

2. Materials and methods

2.1. Materials and instruments

S. nigrum leaves were obtained in September from the farm of medicinal plants, College of Pharmacy, Cairo University, Egypt. Sample of the differentiated plants was authenticated by Prof. Dr. Mounir Abd El-Ghany and deposited at the Herbarium of Cairo University with recording number CAI 343215.

Standard solasonine and solamargine were gifts from Dr. Ashgan Zaki, professor of Pharmacognosy, College of Pharmacy, Cairo University, which were purchased from Sigma Co. (Karlsruhe, Germany). Media ingredients and growth hormones for differentiation and in vitro glycoalkaloids formation are tissue culture grade from Sigma Co. Analysis solvents were high performance liquid chromatography (HPLC) class.

HPLC apparatus was Hewlett Packard 1050 with UV detector. The analyses were carried out at wavelength 210 nm. Column used was C18 5 μm, 0.4 cm × 25 cm. Flow rate was adjusted to 1 mL/min. Pump pressure was set up at six bars. Column temperature was adjusted at 32 °C.

Olympus light microscope was used with eye piece magnifications 25× and oil objective magnifications 100×.

Spectrophotometer (Novaspec LKB Biochrome, Cambridge, UK) was made in Germany with the technical specifications of wave length range 330–800 nm, band width 7 nm and absorbance range 0.300–2.500.

2.2. Tissue culture study

S. nigrum leaves were sterilized with clorox for 20 min, soaked for seconds in 70% ethanol, and then washed twice with sterile distilled water under sterile conditions. Leaves were cut and aseptically implanted in Murashige and Skoog (MS) media[17] supplemented with phytohormones. The media contained different ratios of Indole-3-butyric acid, 1-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid. The cultures were autoclaved at 121 °C, and then maintained at 26 °C and 16/8 light photoperiod. Subculture on fresh media was performed monthly.

Differentiated shoots were subcultured on MS media containing hormones for two months then transferred to hormone free media for root development. Acclimatization of regenerated plants was carried out according to El-Ashaal et al.[14].

2.3. Glycoalkaloids identification

Qualitative identification of glycoalkaloids from cultures was carried out using high performance thin layer chromatography silica plates. About 50 mg of calli were dried, and then extracted with 96% methanol twice (2 × 100 mL). The extract was concentrated under vacuum. The residue was co-chromatographed against standards glycoalkaloids. Eluting system for the glycoalkaloids was chloroform: methanol: 1% ammonia (2:2:1 v/v). While that for aglycone was benzene: methanol (4:1 v/v).

Quantitative analysis of the glycoalkaloids was carried out using HPLC. Plant materials from callus, regenerated shoots, fruits of the acclimatized in vitro plants and mother field S. nigrum leaves were desiccated at 45 °C. About 10 mg of the desiccated materials was macerated, macerated in 25 mL methyl alcohol (96%) at 50 °C for 3 h, and sequently homogenized in methanol using ultra-turax three times each for 5 min (3 × 15 mL). The collective extracts were concentrated under reduced pressure and temperature. The deposited test materials in addition to authentic glycoalkaloids were solved in methyl alcohol (1:1 w/v) and filtered using 0.45 μm Millipore filter. The samples were analyzed using HPLC in triplicates. The injection volume ranged 0.25–1.00 μL depending on the concentration of each sample. Mobile phase (40% methanol) was utilized in isocratic mode. The adapted wave length was 210 nm, flow rate 1 mL/min and temperature was regulated at 40 °C. Standard curves for authentics were plotted. Glycoalkaloids concentrations were calculated by comparing percentage peak areas of the samples with that of authentics.

For solasonine, the obtained control function is: \(Y = 45.55X – 44.25\), with \(r = 0.972\). Also, for α-solamine, the control function is: \(Y = 5.281X + 2.15\), with \(r = 0.994\). The control function for solamargine is: \(Y = –102.4X + 41.83\), with \(r = 0.967\). Finally, the control function for solanidine is: \(Y = 23.03X – 0.229\), with \(r = 0.999\).

Regarding extraction of in vitro produced glycoalkaloids, calli were dried at a temperature of 45 °C, crushed in coarse powder, and then macerated in 5% acetic acid twice. The macerate were filtrated and the filtrated extract was concentrated using vacuum. The concentrate was handled with ammonium hydroxide till pH 14, then pure glycoalkaloids were precipitated upon cooling.

2.4. Biological examination

2.4.1. Mice

Clostridium difficile infection strain of Swiss albino male mice
(20–25 g) was purchased from Research Institute of Theodor Bilharz, Cairo, Egypt which were fed on water as well as standard pellet diet ad libitum (El-Kahira Company for Oil and Soap, Cairo, Egypt). All experimental procedures involving animals were conducted in accordance to the approved guidelines of the Ethical Committee of the National Research Center for use and care of experimental animals.

2.4.2. Homogenization of hepatic tissue
Homogenization of hepatic tissue was carried out using saline solution (1:10 w/v) for the determination of succinate and lactate dehydrogenases (SDH and LDH), lipid peroxide (MDA), glutathione (GSH), vitamins C and E, glucose-6-phosphatase (G-6-Pase), acid phosphatase (AP) and 5′-nucleotidase.

2.4.3. Enzymes extraction method of urea
Mice liver was immediately separated, dried and assessed, and then 4.5 mL of 0.1% hexadecyltrimethyl ammonium bromide was used in the homogenization. The process of homogenization was occurred at 0 °C, centrifuged at 4500 r/min for 10 min at 2 °C. Then the supernatant was separated and preserved at 0 °C and applied for the determination of enzymes. Protein was precipitated from the substrate (enzyme source), by the addition of 5 mL 0.5 mol/L HC1O4, the determination of enzymes. Protein was isolated. The analytical procedures were carried out using the fluid of supernatant.

2.4.4. Doses and route of administration
Glycoalkaloids isolated from S. nigrum cultures were given for 8 weeks along with infection at doses 8 and 16 mg/kg i.p. which were equivalent to 1/4 and 1/2 LD50[12].

2.4.5. Mice grouping
Six groups of six mice each were obtained and classified. Group 1 served as control group. In Groups 2 and 3, normal mice were administrated with 8 and 16 mg/kg glycoalkaloids daily for 8 successive weeks, respectively. Mice in Group 4 were infected with S. mansoni. In Groups 5 and 6, infected mice simultaneously were remediated with glycoalkaloids (8 and 16 mg/kg) for 8 weeks. Post remediation mice were anesthetized with diethyl ether; blood was obtained by cutting sub-tongual vein and centrifuged at 4000 r/min for 15 min; serum was separated and kept at –80 °C for determination of liver enzymes, including aspartate and alanine aminotransferases (AST and ALT) as well as alkaline phosphatase (ALP).

2.5. Biomarkers assessment

2.5.1. Specific biomarker enzymes for cell organelles and total protein content
The activity of SDH enzyme is determined by measuring formazan of 2-p-iodophenyl-3-p-nitropheryl-5-phenyltetrazolium chloride (INT) which is resulted from the decrease in flavin adenine dinucleotide connected with a reduction of INT by spectrophotometric method at 490 nm[18].

The activity of LDH enzyme is assayed by measuring formazan of INT which is resulted from the decrease in nucleoside derived amino acids associated with the reduction in phenazine methosulfate colorimetrically at 503 nm[19].

G-6-Pase, acid phosphatase and 5′-nucleotidase were determined by the assessment of the release of inorganic phosphorus colorimetrically at 660 nm[20-22].

2.5.2. Hepatic function enzyme activities
AST and ALT were evaluated by the described method[23], where oxaloacetate and pyruvate were measured colorimetrically at 520 nm. Alkaline phosphatase stimulated phosphate group conveyed from 4-nitrophosphatase to 2-amino-2-methyl-1-propanol and released 4-nitrophenol. The elaborated color was measured at 510 nm[24].

2.5.3. Markers for oxidative damage
The oxidation of polyunsaturated fatty acids resulted in malondialdehyde which was evaluated according to Mohamed et al.[25]. The concentration of MDA is calculated based on the extinction coefficient 1.56 × 10^5 mol/L·cm^-1 and measured at 535 nm.

Glutathione was demonstrated using pithiobis-2-nitrobenzoic acid in phosphate buffer and the formed color was measured at 412 nm[26]. Folin reagent was used in the estimation of vitamin C and the elaborated color was measured at 560 nm[27]. In addition, vitamin E was assessed using spectrophotometric method[28].

2.5.4. Enzymes activity of urea
Urea enzymes were measured by the method of Ibarra-Gonzále et al.[29], which is the developed method of Morris[30]. Control was used by deactivation of tissue homogenate (100 °C for 10 min), beside sample of blank. The micromoles of the disappearance of substrate or the created product/mg protein/h at 38 °C is known as specific activity.

Ornithine aminotransferase (OAT) enzyme activity was demonstrated spectrophotometrically at 410 nm by measurement of citrulline.

Argininosuccinate synthetase (ASS) enzyme activity was determined through the assessment of un-reacted citrulline spectrophotometrically at 410 nm.

Argininosuccinate lyase (ASL) enzyme broke down arginine to urea prior liver enzyme addition.

The activity of arginase enzyme was estimated through measuring the released ammonia.

2.6. Histological investigation
Liver tissue slices were fixed in 10% buffer formalin. After fixation, paraffin 4 pm thick sections were taken and stained by haematoxylin and eosin[31].

2.7. Data analysis

2.7.1. Statistical analysis
Data were analyzed using SPSS version 10.0 (One-way ANOVA), coupled with co-state computer program, where unshared letters are significant at P ≤ 0.05.

2.7.2. Percentage of changes and improvements
Percentages of changes and improvements were calculated with the following formulas, respectively:

% Change = (Mean of control – Mean of test)/Mean of control × 100
% Improvement = (Mean of infected – Mean of treated)/Mean of control × 100
2.8. Mutation examination

For evaluating the different mutagenic end points, samples were collected 24 h after the last treatment. For examination of chromosomal abnormalities in bone marrow and spleen cells, animals were injected i.p. with colchicine 2–3 h before samples collecting.

2.8.1. Chromosome evaluation in bone marrow and spleen cells (somatic cells)

Maamoun et al.[32] technique was used for chromosome preparations from bone-marrow and spleen cells. The 100-well spread metaphases were analyzed per mouse for evaluating the normal and aberrant chromosomes. Different kinds of aberrations were recorded.

2.8.2. Sperm evaluation (germ cells)

The reported method[33] was used for sperm evaluation. Different sperm abnormalities such as triangle, banana shape, amorphous without hook and coiled tail were recorded.

2.8.3. Data evaluation

Data analysis and statistical evaluation of the DNA damage were performed using t-test. The significance of the results was between the negative control and infected mice with schistosomiasis worm as well as between infected mice with schistosomiasis worm plus glycoalkaloids extracted from S. nigrum against infected mice.

The DNA protective activity of the glycoalkaloids was calculated using the following equation[34]:

\[
\% \text{ Inhibition} = \left(1 - \frac{\text{Glycoalkaloids and schistosomiasis worm} - \text{Control}}{\text{Schistosomiasis worm} - \text{Control}}\right) \times 100
\]

3. Results

3.1. Initiation of callus, differentiation and glycoalkaloids evaluation

The present results showed that callus cultures were initiated in MS media contained Indole-3-butyric acid as cytokinin and 2,4-dichlorophenoxyacetic acid as auxin at the same proportion (1:1). Meanwhile, differentiation was achieved on MS media contained Indole-3-butyric acid as cytokinin and 1-naphthaleneacetic acid as auxin also at the same proportion (1:1) (Figures 1 and 2). Flowering was observed in some cultures. Roots were developed in MS cultures contained basal nutrients and devoid of growth hormones (Figure 3). Acclimatization gave rise to whole regenerated plant with fruits in vivo. The biosynthesized glycoalkaloids were endotoxin free. Qualitative high performance thin layer chromatography chromatographic analysis of callus and shoots methanolic extract indicated the presence of glycoalkaloids spots that gave orange color with Dragendorff’s reagent corresponding to standard solasonine, solanine, solamargine and solanidine alkaloids. Quantitative HPLC assay (Figure 4) for mother leaves, callus, shoots and in vitro derived fruits revealed the success in biosynthesis of solasonine, solanine and solamargine glycoalkaloids in addition to solanidine at increasing concentrations with respect to original plant. Table 1 and Figure 4 showed that solanine was the predominant glycoalkaloid produced in the cultures. The results revealed the presence of solanidine aglycone in cultures while it was absent in intact parent plant. HPLC analysis also showed that in vitro glycoalkaloids were biosynthesized in much higher concentrations than parent plant. Table 1 showed that the concentrations were 1.868, 2.797 and 25.190 folds of the concentrations of the mother plant for solasonine, solanine and solamargine, respectively regarding callus cultures. Concerning shoots, the concentrations were 1.833, 3.124 and 58.861 folds. The increments of total glycoalkaloids for callus and shoots were 2.63 and 2.74 folds, respectively comparing with intact mother leaves derived glycoalkaloids.

Table 1

| Glycoalkaloid | Mi | Ca | Rsh | Rf |
|---------------|----|----|-----|----|
| Solasonine    | 1.382 | 2.582 | 2.534 | 0.644 |
| Solanine      | 2.068 | 5.785 | 6.460 | 6.638 |
| Solamargine   | 0.0079 | 0.1990 | 0.4650 | 0.1540 |
| Solanidine    | –   | 0.5380 | –   | 0.2380 |

Mi: Mother derived leaves; Ca: Callus; Rsh: Regenerated shoots; Rf: Regenerated plants fruits.
hepatic enzyme. However, infection of mice with glycoalkaloids showed no detectable changes in activities of AST, ALT and ALP, respectively. A statistically elevation in hepatic marker enzymes by 41.65%, 42.16% and 45.24% for AST, ALT and ALP, respectively. Eight weeks upon using glycoalkaloids at dose 16 mg/kg (Table 3).

Values are mean ± SD, n = 6. Statistical analysis is carried out by independent t-test. a: P < 0.05, b: P < 0.001.

3.2. Biomarkers assays

Table 2 shows the effect of S. nigrum glycoalkaloids remediation on hepatic enzymes. Healthy control animals i.p. injected with glycoalkaloids showed no detectable changes in activities of hepatic enzyme. However, infection of mice with S. mansoni exhibited statistically elevation in hepatic marker enzymes by 41.65%, 42.16% and 45.24% for AST, ALT and ALP, respectively. Intrapertioneally treatment of infected mice glycoalkaloids for 8 weeks at dose 8 mg/kg demonstrated improvement percentages reached to 34.64%, 36.61% and 42.95%, respectively (Table 3).

Table 4 indicates insignificant change on biomarkers enzymes, oxidative stress biomarkers and urea cycle enzymes after S. nigrum glycoalkaloids treatment (%).

With respect to oxidative stress biomarkers, Table 5 shows that glycoalkaloids treated normal mice indicated insignificant differences in MDA, GSH, vitamins C and E. Although mice infected with S. mansoni demonstrated statistically elevation in MDA by 339.53%, GSH, vitamins C and E exhibited significant reduction reached to 48.75%, 55.43% and 65.39%, respectively. Table 3 illustrated that treatment of glycoalkaloids to infected mice at dose 8 mg/kg for 8 weeks recorded amelioration in MDA, GSH, vitamins C and E by 230.23%, 28.48%, 36.41% and 44.98%, successively. However, the percentage of improvement recorded 323.25%, 43.08%, 63.36% and 65.05%, respectively upon using dose 16 mg/kg.

- Oogram, worm burden and ova count in both liver and intestine of infected mice received 8 and 16 mg/kg glycoalkaloids for 8 weeks demonstrated statistically increment in the activity of OAT (82.56%), however ASS, ASL and arginase activities reached to 48.75%, 55.43% and 65.39%, successively for OAT, ASS, ASL and arginase enzyme activities, while 16 mg/kg treatment recorded percentages of improvement 84.37%, 57.60%, 51.02% and 65.38%, respectively (Table 3).

Table 5 shows the effect of S. nigrum glycoalkaloids treatment on liver function enzymes in S. mansoni infected and infected-treated mice (μmol/min/mg protein).

| Parameters     | Improvement (8 mg/kg) | Improvement (16 mg/kg) |
|----------------|-----------------------|------------------------|
| AST            | 22.28                 | 34.64                  |
| ALT            | 31.88                 | 36.61                  |
| ALP            | 33.44                 | 42.95                  |
| SDH            | 16.80                 | 41.17                  |
| LDH            | 19.74                 | 29.75                  |
| G-6-Pase       | 17.36                 | 25.65                  |
| AP             | 2.59                  | 14.28                  |
| 5’-nucleotidase| 86.44                 | 103.14                 |
| MDA            | 230.23                | 323.25                 |
| GSH            | 28.48                 | 43.08                  |
| Vitamin C      | 36.41                 | 63.36                  |
| Vitamin E      | 44.98                 | 65.05                  |
| OAT            | 60.32                 | 84.37                  |
| ASS            | 41.60                 | 57.60                  |
| ASL            | 43.46                 | 51.02                  |
| Arginase       | 48.69                 | 65.38                  |

With respect to oxidative stress biomarkers, Table 6 demonstrated that remediation of healthy mice with glycoalkaloids showed statistically no difference in enzyme activities comparing with healthy mice not received glycoalkaloids. Infected mice with S. mansoni demonstrated statistically increment in the activity of OAT (82.56%), however ASS, ASL and arginase activities declared statistically inhibition reached to 53.60%, 57.94% and 72.02%, successively. Treatment of infected mice with 8 mg/kg glycoalkaloids recorded amelioration by 60.32%, 41.60%, 43.46% and 48.69%, successively for OAT, ASS, ASL and arginase enzyme activities, while 16 mg/kg treatment recorded percentages of improvement 84.37%, 57.60%, 51.02% and 65.38%, respectively (Table 5).

- Oogram, worm burden and ova count in both liver and intestine of infected mice received 8 and 16 mg/kg glycoalkaloids for 8 weeks exhibited significant dose dependent decrease in these parasitological indices comparing to untreated-infected mice (Tables 7–9).
3.3. Histopathological study

Histopathological investigation showed that treatment of *S. nigrum* glycoalkaloids at the chosen doses had no adverse effect on normal mice. Meanwhile, the deposited and trapped eggs in hepatic perisinusoidal spaces of infected mice induced severe hepatic granulomatous inflammation that caused disorganization of the hepatic strands and lobular structure. Inflammatory response appears in form of infiltrate of inflammatory cells, vacuolation of cytoplasm and hepatocytes degeneration (Figure 5). The results illustrated improvement in liver architecture after the two doses of 8 and 16 mg/kg glycoalkaloids treatment to infected mice for 8 weeks. The improvement was dose dependent and showed disintegrated eggs in granuloma of liver sections, lesions decreased and granuloma became smaller (Figure 6).

Table 4
Effect of *S. nigrum* glycoalkaloids treatment on cell organelles markers enzymes in *S. mansoni* infected and infected-treated mice (μmol/min/ mg protein).

| Groups | Parameters | SDH % Change | LDH % Change | G-6-Pase % Change | AP % Change | 5'-nucleotidase % Change |
|--------|------------|--------------|--------------|-------------------|-------------|-------------------------|
| Group 1 |            | 1.19 ± 0.16  | –            | 349.60 ± 19.52    | –           | 98.45 ± 6.10            |
| Group 2 |            | 1.12 ± 0.09  | –3.88        | 344.59 ± 17.60    | –1.43       | 99.00 ± 6.60            |
| Group 3 |            | 1.16 ± 0.09  | –2.52        | 346.16 ± 12.67    | –0.98       | 98.99 ± 7.78            |
| Group 4 |            | 0.69 ± 0.08³ | –42.01       | 250.00 ± 15.22²  | –34.21      | 73.08 ± 9.10¹           |
| Group 5 |            | 0.89 ± 0.08³ | –25.21       | 299.00 ± 18.72²  | –14.47      | 90.18 ± 8.18³           |
| Group 6 |            | 1.18 ± 0.02  | –0.84        | 334.00 ± 8.00²    | –4.46       | 98.33 ± 0.36            |

Values are mean ± SD, *n* = 6. Statistical analysis is carried out by independent *t*-test. ³: *P* < 0.05; ²: *P* < 0.001.

Table 5
Effect of *S. nigrum* glycoalkaloids treatment on oxidative stress markers in *S. mansoni* infected and infected-treated mice.

| Groups | Parameters | MDA % Change | GSH % Change | Vitamin C % Change | Vitamin E % Change |
|--------|------------|--------------|--------------|--------------------|--------------------|
| Group 1 |            | 0.43 ± 0.10  | 48.79 ± 3.60 | 9.20 ± 0.61        | 2.89 ± 0.19        |
| Group 2 |            | 0.46 ± 0.04  | 47.00 ± 3.10 | 9.90 ± 0.44        | 2.85 ± 0.07        |
| Group 3 |            | 0.41 ± 0.03  | 48.19 ± 6.40 | 9.44 ± 1.00        | 2.87 ± 0.11        |
| Group 4 |            | 1.89 ± 0.10² | 25.10 ± 2.00²| 4.10 ± 0.32³       | 1.00 ± 0.18³       |
| Group 5 |            | 0.90 ± 0.03³ | 39.00 ± 0.98³| 7.45 ± 0.10³       | 2.30 ± 0.11³       |
| Group 6 |            | 0.50 ± 0.02  | 46.12 ± 2.45 | 9.93 ± 0.11        | 2.88 ± 0.03        |

Values are mean ± SD, *n* = 6. Statistical analysis is carried out by independent *t*-test. ³: *P* < 0.05; ²: *P* < 0.001; ³: *P* < 0.0001. Data are expressed as μg/mg protein for GSH, Vitamin C and E, and μmol/mg protein for MDA.

Table 6
Effect of *S. nigrum* glycoalkaloids treatment on urea cycle enzymes in liver of *S. mansoni* infected and infected-treated mice (μmol/min/mg protein).

| Groups | Parameters | OAT % Change | ASS % Change | ASL % Change | Arginase % Change |
|--------|------------|--------------|--------------|--------------|-------------------|
| Group 1 |            | 4.99 ± 0.67  | 1.25 ± 0.20  | 22.09 ± 2.78 | 46.80 ± 4.00      |
| Group 2 |            | 4.59 ± 0.69  | –8.01        | 22.34 ± 3.89 | 47.30 ± 4.30      |
| Group 3 |            | 4.70 ± 0.45  | 5.81         | 23.07 ± 3.18 | 46.00 ± 6.13      |
| Group 4 |            | 9.11 ± 0.19³ | 82.56³       | 9.29 ± 1.00³ | 13.09 ± 1.22³     |
| Group 5 |            | 6.10 ± 1.00³ | 22.24³       | 18.89 ± 2.04³ | 35.88 ± 6.23³     |
| Group 6 |            | 4.90 ± 1.05  | –1.80        | 20.56 ± 3.95 | 43.69 ± 5.09      |

Values are mean ± SD, *n* = 6. Statistical analysis is carried out by independent *t*-test. ³: *P* < 0.05; ²: *P* < 0.001; ³: *P* < 0.0001.

Figure 4. HPLC analyses of glycoalkaloids from callus (A) and regenerated fruits (B) of *S. nigrum* cultures.

1: Solasonine; 2: Solanine; 3: Solamargine; 4: Solanidine
Table 7
Oogram in infected and infected-treated in mice with *S. nigrum* glycoalkaloids.

| Groups Oogram | % Change |
|---------------|----------|
| Infected Dead | 5.00 ± 0.55 |
| Immature      | 33.20 ± 5.90 |
| Mature        | 50.00 ± 9.06 |
| Infected + treated (8 mg/kg) Dead | 30.00 ± 3.33 | +500.00% |
| Immature      | 60.00 ± 7.12 | +80.72% |
| Mature        | 10.05 ± 9.30 | –79.90% |
| Infected + treated (16 mg/kg) Dead | 40.90 ± 4.82 | +718.00% |
| Immature      | 45.00 ± 10.84 | +35.54% |
| Mature        | 10.77 ± 1.00 | –78.46% |

Values are mean ± SD, *n* = 6. Statistical analysis is carried out by independent *t*-test. *a*: *P* < 0.05; *b*: *P* < 0.001; *c*: *P* < 0.000 1.

Table 8
Worm count in infected and infected-treated in mice with *S. nigrum* glycoalkaloids.

| Parameters | Worm count | % Change |
|------------|------------|----------|
| Infected Female | 3.20 ± 1.92 |
| Male    | 9.10 ± 0.60 |
| Couple  | 9.00 ± 1.90 |
| Infected + treated (8 mg/kg) Female | 3.30 ± 0.54 | +3.12% |
| Male    | 3.00 ± 0.74 | –67.03% |
| Couple  | 4.60 ± 0.59 | +48.88% |
| Infected + treated (16 mg/kg) Female | 2.10 ± 0.22 | –34.37% |
| Male    | 2.06 ± 0.47 | –77.36% |
| Couple  | 3.00 ± 0.03 | –66.66% |

Values are mean ± SD, *n* = 6. Statistical analysis is carried out by independent *t*-test. *a*: *P* < 0.05; *b*: *P* < 0.001; *c*: *P* < 0.000 1.

3.4. Chromosomal analysis

3.4.1. Chromosomal aberrations in somatic cells

Tables 10 and 11 showed the different percentage of aberrations in all tested groups. Glycoalkaloids-treated group showed no statistically difference than the control group. While glycoalkaloids-treated infected groups with schistosomiasis showed a statistically significant (*P < 0.01*) inhibition in aberrant chromosomes comparing to infected groups alone. The percentage of inhibition of chromosome damage was dose dependent in bone marrow and spleen cells (Tables 10 and 11).

3.4.2. Sperm-shape abnormalities

The percentage of sperm abnormalities in glycoalkaloids group was nearly close to the control group (Table 12). Mean percentage of sperms were (8.14 ± 0.64)% and (7.66 ± 0.80)% with 8 and 16
Each group contains five animals.

| Treatments (mg/kg body weight) | Time (weeks) | No. of different types of metaphases | % Inhibition excluding gaps |
|--------------------------------|--------------|-------------------------------------|-----------------------------|
|                                |              | Including gaps | Excluding gaps | Gap | Fragments and/or breaks | Deletions | CF | MA | Po |
| I. Control                   | 26           | 5.20 ± 0.66    | 0.00 ± 0.63    | 11  | 13                       | 2         | 0  | 0  | 0  |
| II. Glycoalkaloids           | 8            | 5.40 ± 0.50    | 3.00 ± 0.45    | 12  | 12                       | 3         | 0  | 0  | 0  |
|                             | 16           | 6.40 ± 0.63    | 3.60 ± 0.93    | 14  | 15                       | 3         | 0  | 0  | 0  |
| III. Infected + glycoalkaloids | 86           | 13.20 ± 0.93   | 8.80 ± 0.40    | 22  | 28                       | 8         | 3  | 4  | 1  |
| IV. Infected + glycoalkaloids | 8            | 9.20 ± 0.83    | 6.20 ± 0.50    | 15  | 9                        | 6         | 2  | 3  | 1  |
|                             | 16           | 8.00 ± 0.75    | 5.40 ± 0.45    | 13  | 16                       | 7         | 0  | 3  | 1  | 59 |

There are 500 metaphases examined in total (100 metaphase/animal, 5 animals/group). CF: Centric fusions; MA: Multiple aberrations; Po: Polyploidy. *: Significant difference between infected group and control group at P < 0.01; †: Significant difference between infected group treated with the extract compared to infected group at P < 0.01 (t-test).

Table 11 Percentage of chromosomal aberrations and the number of the different types of aberrations in the mouse spleen cells infected with schistosomiasis before and after treatment with *S. nigrum* glycoalkaloids.

| Treatments (mg/kg body weight) | Time (weeks) | No. of sperm examined | Abnormal sperms | No. of different types of sperm abnormalities | % Inhibition |
|--------------------------------|--------------|-----------------------|-----------------|-----------------------------------------------|--------------|
|                                |              |                       | No. | Mean ± SE (%) | Triangular | Banana shape | Amorphous | Without hook | Coiled tail |            |
| I. Control                     | 27           | 5.40 ± 0.50           | 17  | 2.80 ± 0.50   | 13           | 10          | 4          | 0          | 0          | 0          |
| II. Glycoalkaloids             | 8            | 6.00 ± 0.93           | 13  | 3.40 ± 0.66   | 12           | 15          | 5          | 0          | 0          | 0          |
|                                | 16           | 6.60 ± 0.85           | 12  | 4.20 ± 0.50   | 23           | 30          | 7          | 2          | 6          | 2          |
| III. Infected + glycoalkaloids | 8            | 9.00 ± 0.38           | 17  | 7.40 ± 0.80   | 24           | 22          | 6          | 1          | 4          | 2          |
| IV. Infected + glycoalkaloids  | 16           | 9.80 ± 0.63           | 16  | 6.60 ± 0.60   | 21           | 21          | 5          | 1          | 5          | 1          | 43 |

There are 500 metaphases examined in total (100 metaphase/animal, 5 animals/group). CF: Centric fusions; MA: Multiple aberrations; Po: Polyploidy. *: Significant difference between infected group and control group at P < 0.01; †: Significant difference between infected group treated with the extract compared to infected group at P < 0.01 and P < 0.05, respectively (t-test).

Table 12 Percentage of inhibitory index of sperm abnormalities after treatment of schistosomiasis infected group with glycoalkaloids.

| Treatments (mg/kg body weight) | Time (weeks) | No. of different types of sperm abnormalities | % Inhibition |
|--------------------------------|--------------|-----------------------------------------------|--------------|
|                                |              | No. | Mean ± SE (%) | Triangular | Banana shape | Amorphous | Without hook | Coiled tail |            |
| I. Control                     | 27           | 5.182 | 2.95 ± 0.58 | 143       | 8           | 67       | 22          | 15         | –          |
| II. Glycoalkaloids             | 8            | 5.121 | 3.86 ± 0.78 | 45        | 6           | 90       | 29          | 23         | 8          |
|                                | 16           | 5.094 | 4.06 ± 0.70 | 51        | 10          | 87       | 25          | 34         | –          |
| III. Infected + glycoalkaloids | 8            | 5.173 | 5.16 ± 0.82 | 147       | 90          | 148      | 112         | 56         | –          |
| IV. Infected + glycoalkaloids  | 16           | 5.158 | 4.76 ± 0.80 | 104       | 62          | 136      | 71          | 47         | 33         |

Each group contains five animals. *: Significant difference between infected group and control group at P < 0.01; †: Significant difference between infected group treated with the extract compared to infected group at P < 0.05 and P < 0.01, respectively (t-test).

mg/kg body weight of glycoalkaloids administered in the same time of mice infection with schistosomiasis for 8 weeks, respectively comparing with (10.69 ± 0.82)% for infected mice. The reduction in sperm abnormalities was dose dependent. The percentage of inhibitory index increased as the dose of treatment increased (Table 12).

4. Discussion

The present results illustrated that phytohormones play substantial role in callus evolution and cell proliferation to shoots. The optimum cytokinin: auxin ratios were (1:1) for both calli and differentiation cultures. Meanwhile, phytohormones were not essential for root development. On contrast to the present results, it was reported that auxins and cytokinins were essential for root development in *S. torval* cultures [35]. Shoots of *S. nigrum* cultures were found to grow successfully in MS media containing Indole-3-butyric acid and 1-naphthaleneacetic acid, but at cytokinin: auxin ratio (1:2) with roots in hormone free MS medium [14].

Current data revealed that solanarine was biosynthesized at the highest glycoalkaloids concentration regarding callus and regenerated plants followed by solasonine and solamargine. This is consonant with researches reported that solanine is the glycoalkaloid with the highest concentrations in *S. nigrum* fruits in addition to solasonine and solamargine [36]. The biosynthesis of solanidine in cultures might be due to partial hydrolysis of solanarine. The *in vitro* glycoalkaloids production in increasing concentrations than intact derived plant was remarkable outcome of our study.
Regarding callus and differentiated shoots, solamargine displayed the highest increment with respect to original derived plant (25.14, 58.69 folds) followed by solanine (2.798, 3.124 folds) and finally solasonine (1.868, 1.833 folds). The increments of total glycoalkaloids were 2.63 and 2.74 folds, respectively. This could be attributed to selection of high strain yield mother plant and optimization of culture conditions. Berberine alkaloid was produced from in vitro culture of Thalictrum minor 1.000 folds than original plant[13]. In vitro production of solasodine from cultures of S. nigrum (0.142 mg/g) in higher yields than parent plant (0.0798 mg/g) which was equal to 1.78 folds was also reported[15]. High yield of glycoalkaloids of solanidine series from Solanum tuberosum culture, reached 1.44 and 3.88 folds of the concentration of mother plant from calli and shoots, respectively were reported from Solanum tuberosum cultures[16]. In spite that wild Solanum species may contain high glycoalkaloids content, and are widely used in breeding studies that may result in high levels of glycoalkaloids. Unfortunately, the levels of glycoalkaloids might be extremely changed[12]. So, the current study is of great importance for the potential role of in vitro cultures for producing glycoalkaloids in such high yield from S. nigrum plant.

The current results indicated significant increase in oxidative stress biomarkers as represented by malondialdehyde, while there was a significant decrease in glutathione, vitamin E and C in mice infected with S. mansoni. These results declared markedly antioxidant impaired system by infection since glutathione depletion represented as a marker of impaired immune system defense machinery, utilization of more antioxidant by the liver cells as a consequence of oxidative stress[37]. This is in concomitant with reports declared that infection with S. mansoni is associated with oxidative stress leading to elevation in reactive oxygen species that in turn leading to increment in lipid peroxidation, which is used as powerful tool for oxidative stress assay associated with chronic diseases[25,38].

Considering vitamins C and E, significant diminution was recorded in mice infected with S. mansoni. These results are in agreement with studies found peroxyl radical scavenging activity of ascorbate and hence the enzymes and vitamins levels are significantly decreased during this process[39]. As well, the decrease of vitamin E post bilharzia infection may be explained on the basis that this vitamin is regard as a soluble antioxidant, which plays a principle role in cell membranes protection against free radicals and hence preserves cell structure and functions. In addition, vitamin E protects hepatic cells against toxicity related injury[40].

With respect to hepatic function enzymes, the present results declared significant elevation in the activities liver enzyme in mice infected with S. mansoni. In this concern, significant elevation in AST, ALT and ALP enzyme activities post S. mansoni infection was reported[41]. The authors related these elevations to the enzymes leakage to the blood stream as a consequence of free radical by infection, which may cause mitochondrial membrane destruction and increasing of cell membrane permeability leading to discharging of enzymes into circulation.

Regarding to SDH enzyme activity, the present results illustrated SDH significant inhibition 8 weeks post infection. This inhibition in SDH enzyme activity may be due to accumulation of toxins elaborated by schistosomal infection within the mitochondria of hepatic cells which in turn affected on enzyme activities[41]. On the other hand, the inhibition in LDH enzyme activity in S. mansoni parasitic infection may be attributed to larvae infection caused hepatic tissue damage, led to enzyme leakage to the circulation as well as agitation and low oxygen level as a results of metabolic toxic products of the parasitic worm[42]. Moreover, the present results demonstrated significant decrease in G-6-Pase enzyme activity post S. mansoni infection. The inhibition in enzyme activity may be due to deterioration in glycogen metabolism[43]. While, the present results illustrated significant elevation in AP activity post parasitic infection. This result is in concomitant with previous studies attributed this increment to lysosomes deflection and/or to destructive metabolism by the elevation of worm and eggs toxins since AP is considered as lysosomal enzyme and during infection all the lysosomal enzymes are enhanced due to destructive tissue initiated phagocytosis[44].

The present data also declared statistically increment in 5'-nucleotidase activity post infected mice with S. mansoni. This increase in enzyme activity may be related to activation in plasma membrane transport function where the enzyme localized at liver cell membrane as well as acceleration of nucleic acid metabolism, since 5'-nucleotidase stimulated the destruction of nucleic acid nucleotides[45].

The influence of S. mansoni infection on urea cycle enzyme activities declared that OAT showed a significant increase two-month post infection, where as a significant decrease was found in ASS activity post parasitic infection. Also, ASL and arginase enzyme activities demonstrated extensive inhibitory activity two months post infection comparing to normal control mice. These results are in accordance to authors who found that parasitic infection resulted in deterioration in the metabolism of protein and/or the synthesis of enzymes, so disturbances of the different pathways of metabolism included enzymes regulation of urea. Also, OAT is localized within mitochondria, and during parasitic infection, toxins are accumulated within the mitochondria which become swollen and disrupted leading to OAT discharge into the circulation[46]. The incoordination between OAT enzyme and cytoplasmic arginase is considered as a pathological status rather than adaptive response during parasitic disease. The present data ascertained by observation showed that S. mansoni performed disturbances in enzyme activities of urea associated with fluctuation in the concentrations of enzyme[47]. Also, the significant decrease in arginase levels may be due to imbalance between synthetic machinery and rates of degradation as results of elaborated toxins by parasite.

Significant reduction was found in carbamoyl phosphate synthetase, OAT as well as in the level of urea 10 weeks post infection. This may be due to S. mansoni eggs induced granuloma and inflammatory cells which may be attributed to the decrease in these enzyme activities or may be due to granuloma cause enlargement of liver associated with reduction in the number of liver cell containing enzymes of urea cycle. In addition, there is a possibility that the suppression of carbamoyl phosphate synthetase which is considered as a one of rate-limiting step in urea cycle synthesis leads to decrease in the enzymes synthesis and activity of urea cycle[48].

The current outcomes indicated marked amelioration in
biochemical and antioxidant parameters under investigation of infected mice that documented by enhancement in histopathological examination at the cellular level after *S. nigrum*, glycoalkaloids *i.p.* injection. These improvements were dose dependent. The results also illustrated that schistosomal infection was coupled by oxidative stress and egg induced liver inflammation. Oxidative stress is pronounced from the elevation of lipid peroxidation and decreasing vitamins activities (Table 5). Liver histopathological examination illustrated inflammation combined by lesions, and liver granuloma causing intense liver inflammation and pathological scarring (Figure 5). These observations are consistent with studies documented that inflammation induced by *Schistosoma haematobium* infection may lead to inducible nitric oxide synthase-dependent DNA damage[4]. Infection with *S. mansoni* was found to cause a severe hepatic granulomatous inflammatory response[2,49].

So, the marked enhancement after glycoalkaloids administration in our work might be due to the observed antioxidant and antiinflammatory activities owing to the presence of solasonine, solamargine and solanine. Restoring vitamins and lipid peroxidation levels to approximately their normal levels in the current study is indicative of antioxidant potency of the isolated glycoalkaloids (Table 5). This finding is compatible with finding owed the antioxidant activity of *S. lycocarpum* to solasodine glycosides including solasonine and solamargine[50]. Besides, solanine and other glycoalkaloids were reported to exhibit antiinflammatory activity[51].

The antiinflammatory activity of isolated glycoalkaloids in the current results was estimated through histological improvement regarding number of lesions, granuloma size and disintegrated eggs in glycoalkaloids treated groups comparing to infected one (Figure 6). The present improvements in liver inflammation are documented by liver histopathological analysis studies which revealed that *S. nigrum* extract decreased liver lesions incidence. Moreover, the researchers reported that histological study assured that the degree of fibrosis caused by thioacetamide (TAA) treatment was reduced by *S. nigrum* extract by reducing the amount of hydroxyproline and consequently collagen[52].

The current improvements in biomarkers under investigation are also documented by parasitological findings which revealed statistically decrease in oogram, worm count as well as ova count in hepatic and intestinal tissues of infected mice (Tables 7–9).

Our results are confirmed by previous reports of *S. nigrum* aqueous fruit extract effectiveness on hepatic marker enzymes and renal function markers in rats administered ethanol. Arulmozh et al. reported that utilization of *S. nigrum* extract restored the diminished levels of AST, ALT, ALP, γ-glutamyl transpeptidase, bilirubin, urea, uric acid and creatinine. They also found that superoxide dismutase, catalase and glutathione peroxidase activities as a marker of antioxidant situation were normalized indicating repair of the hepatic tissue harm resulted from ethanol[53].

In a good agreement with the present finding, clinical trials using polyherbal formulations in which *S. nigrum* is one of the ingredients, have been utilized as hepatoprotective medicament due to its high antioxidant activity[54]. Sub lethal concentration of *S. nigrum* extracts showed potent effect in disturbing snail biomarkers as acid phosphatase and alkaline phosphatase enzymes which may make them unsuitable physiologically for growing schistosoma parasite[55]. Binary combination of *S. nigrum* and *Iris pseudacorus* showed molluscsidal and cercaricidal efficiency toward * Biomphalaria alexandrina* and *S. mansoni* cercariae, respectively. Meanwhile, pre-treatment of mice with varied concentration of crude water extract of *S. nigrum* performed statistically significant decrease in permeation and infectivity of *S. mansoni* cercariae[56].

In this context, it was found that extracts of *Solanum zeybrilifolium* as well as isolated solamargine, displayed elevated molluscsidal activity and low mortality against non-target species (fish and macro invertebrate). While in laboratory conditions, solamargine and β-solamarine at lethal concentration caused 100% mortality of cercariae[57].

Concerning mutation study, the present data confirmed that *S. mansoni* infection induced significant somatic aberration in mice comparing to control untreated group. The results are in close agreements with findings which are illustrated that schistosomiasis leads to induction of DNA damage in human cell[58]. Schistosomiasis induced oxidative stress might lead to mutation. Oxidative stress induced free radicals that could damage DNA and result in mutation which might progress leading to cancer[4]. Our results show that glycoalkaloids isolated from *S. nigrum* were genotoxic safe and has no genotoxicity hazards. On the contrary, glycoalkaloids have the ability to inhibit the DNA damage in somatic and germ cells in mice that might be a virtue of the induced antioxidant activity observed in our work. In good agreement with these results, glycoalkaloid extract of *S. lycocarpum* not only exerted no genotoxic effect, but also significantly reduced the frequency of aberrations induced by mitomycin C in V79 cells[11]. Solanine, a steroid alkaloid isolated from *S. nigrum* was found to have anti-tumor activity against three tumor cell lines namely, HepG2, SGC-7901, and LS-174 and signs for apoptosis were found[59]. *Solanum xanthocarpum* and *Juniperus communis* extracts had hepatoprotective potential against paracetamol and azithromycin induced liver toxicity due to their synergistic antioxidant properties[60]. Also, the antimutagenic activity of phenol extract of *Solanum melongena* using the salmonella/microsome assay[61].

It is important to know that praziquantel, the famous oral antibilharzia drug, was documented in previous reports in our laboratories to induce chromosomal aberrations at its therapeutic dose that might prolong to next generations in spite of its improvement signs concerning biochemical parameters and histological examination[5,6,62]. Other chemotherapy agents were reported to have severe side effects as hepatotoxicity and cardiac muscle toxicity. Among these agents, Miracil D (thioxanthone derivatives) is taken orally and antimonial compounds which are taken via intravenous or intramuscular routes[63].

In conclusion, the present study could be a good guide for *in vitro* biosynthesis of glycoalkaloids in continual constant pattern. The biological investigation revealed the potency of the separated glycoalkaloids against schistosomiasis infection regarding the improvement in all biomarkers, histological inflammation and oxidation parameters. In addition, alteration of bilharziasis induced genotoxic mutation. The results also illustrated the safety of glycoalkaloids with respect to liver and kidney functions, hepatic
cells structures and DNA chromosomes. Thus, the current study could be a convenient rapprochement for natural anti schistosomiasis medicine and help to control one of the most dangerous parasitic diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This work was funded by National Research Centre (Egypt) to whom the authors are grateful.

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