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Original Research

Study of hepatoprotective properties of protein fractions obtained from leaves of *Tagetes erecta* L. and *Salvia aethiopis* L. plants by gel filtration chromatography on tetrachloromethane (CCl_4) damaged hepatocytes of mice

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Abstract

The study presents data on the action of protein fractions obtained from *Tagetes erecta* L. and *Salvia aethiopis* L. plants by gel filtration chromatography on the hepatocytes of mice damaged by a 10% tetrachloromethane solution. Protein fractions of the third and fourth peaks have weak hepatoprotective properties. Each peak was also separated into several fractions by the method of ion exchange chromatography. The SDS-PAGE revealed fractions, including those with a molecular weight of 23-47 kDa, which probably participate in the protection of hepatocytes.

Keywords: Tagetes erecta L., Salvia Aethiopis L., Imeretian saffron, proteins, gel filtration chromatography, ion exchange chromatography, tetrachloromethane, CCl_4, gel electrophoresis, histomorphology, hepatocytes.

Introduction

The homeland of representatives of the genus Tagetes is the American continent, they distributed in the wild from the USA to Argentina. In Europe and Georgia, the plants of this genus were artificially distributed in the 16th century. In Georgia *T. electra* L (large-flowered form) and *T. patula* L. (small-flowered form). Quite often these forms are considered as synonym species [1;2]. In the Caucasus and Russia they are known as Imeretian saffron (the name is related to the Georgian region Imereti, a region of Georgia situated in the central-western part of the country) [3].

Representatives of the genus Tagetes contain various biologically active compounds, including phenol, topene, benzophene derivatives, triterpenes, alkaloids, flavonoids, carotenoids and others. They are used in folk medicine and cooking [4].

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According to the data of the scientific literature, the compounds present in the species of the genus Tagetes are distinguished by their action against various pathogenic agents of humans and other organisms, including negative effects on the growth of bacteria and fungi [5].

The extract of *T. minuta* leaves suppressed the growth of Gram-negative bacteria and did not affect microorganisms of normal human microflora (*Lactobacillus, Zymomonas, Saccharomyces*). The quercetagenin-7-arabinosyl-galactoside isolated from this plant was shown to be active against pathogenic bacteria [6].

Flavonoid patuletin isolated from the methanolic extract of the *T. patula*, was demonstrated to suppress cervical tumor cells, having a similar action to glycoside patulitrin and phenolic acid. According to the authors, the cytotoxic effect is related to the antioxidant activity. It should be noted that in folk medicine *T. patula* flowers are used as an antitumor [7].

Biologically active compounds present in species of the genus Tagetes are effective against parasites of plants important for agriculture, for example, essential oils obtained from *T. minuta* leaf [8].

Methanolic extract of *T. patula* at concentrations of 5 and 10 mg/mL adversely affected the growth and development of the causal agents of plant diseases *Botrytis cinerea, Fusarium moniliforme* and *Pythium ultimum*. Probably, due to the free radical formation, the extract initiated changes in the cell membranes of fungi, leading to premature ageing of fungal mycelium [9].

Plants of the genus Salvia also contain biologically active compounds. Sage is widespread throughout Europe, and on the North American continent, 13 species of this genus are found in Georgia [10].

Triterpenes isolated from *S. argentea* leaves are characterized by antibacterial action, their effects were similar to those caused by antibiotics [11].

In addition to antibacterial properties, secondary metabolites of *S. argentea* have a wide spectrum of antioxidant, anti-inflammatory, and cytotoxic activities [12].

Triterpenes isolated from *Salvia grossheimii in vitro* inhibited the growth of human cancer cells [13].

Essential oils isolated from the stem of *Salvia microphylla* inhibited the growth and development of bacteria, especially strongly suppressing *Enterobacter cloacae*. In addition, they were characterized by anti-fungal properties, suppressing the growth of *Candida albicans* [14].

The extracts of *Salvia microphylla* Kunth, prevented the development of scopolamine-induced histopathological changes in the prefrontal cortex and hippocampus of the rats, and inhibited the deposition of β-amyloid in brain areas [15]. However, information on the protein composition of these species is absent.

The aim of this study was the identification of biologically active proteins of *Tagetes erecta* L. and *Salvia aethiopis* L. and the investigation of hepatoprotective properties of protein fractions obtained from the leaves of these plants.

Materials and Methods

Plant Material and Extraction

Tagetes erecta L. and *Salvia aethiopis* L. samples were obtained in the eastern region of Georgia (coordinates: 41° 47' 11" N, 45° 1' 20" E and 41°43′21″N 44°47′33″E).

The leaves were washed in distilled water and homogenized with 40% PBS solution in

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a ratio of 1:5. The extract was filtrated and the total protein concentration in the extract was determined using the Lowry method. After, the extract was centrifuged at 3000 rpm for 30 min.

Protein purification

The protein from *Tagetes erecta* L. and *Salvia aethiopis* L. extract was concentrated using precipitation with 90% ammonium sulfate solution. The total protein concentration was determined using the Lowry method [16]. The protein solution was subjected to gel filtration on a Sephadex G75 column equilibrated with 5 mM KH_2PO_4. 150 mM NaCl pH 7.4. Fractions were collected at a flow rate of 1 ml/min.

Next, the protein was purified by ion exchange chromatography on DEAE–Sephadex. The protein was eluted by gradient by 10 mM Tris-HCl pH 8.0/10 mM Tris-HCl, NaCl 0.25 M pH 8. Fractions were collected at a flow rate of 2 ml/min.

Animals

White mice, 4–6 months (average weight of 30 ± 0.05 g), were used in the study. The mice were housed under normal light-dark conditions (12 hours light followed by 12 hours dark) for the entire experiment and had access to food and water, *ad libitum*. The animals were randomly assigned to one of 4 groups:

The first group: control animals, intraperitoneally injected with 5%PBS solution for 30 days.

The second group of animals was intraperitoneally injected with 10% oil CCl_4 solution.

The third group of animals was intraperitoneally injected with 10% oil CCl_4 solution and protein fractions of *Tagetes erecta* L. and *Salvia aethiopis* L., obtained by gel filtration chromatography at a concentration of 0.0537 mg/ml (0.00179 mg protein/g of animal weight).

In the fourth group, we administered intraperitoneally protein fractions of *Tagetes erecta* L. and *Salvia aethiopis* L., obtained by gel filtration chromatography at a concentration of 0.0537 mg/ml.

Histological staining

Van Gieson's Stain of liver extracted from 6 mice was performed for each group. Mice were anesthetized with chloroform; livers were removed and placed in 15% formalin solution. Liver tissues were sliced into coronal sections using a rotary microtome. Images were obtained using AmScope microscope.

Results and discussion

Combined protein fractions of the third and fourth peaks of *Tagetes erecta* L. and *Salvia aethiopis* L. retain their initial concentration for 30 days after the start of the experiment at +4°C within 0.0537 mg/ml, which allows their use in experiments for a long time (Figure 1 A and B).

DOI: 10.56580/GEOMEDI47

Figure 1 A and B. Third and fourth peaks of *Tagetes erecta* L. and *Salvia aethiopis* L. protein fractions: A. Before the start of the experiment and B. After the end of the experiment.

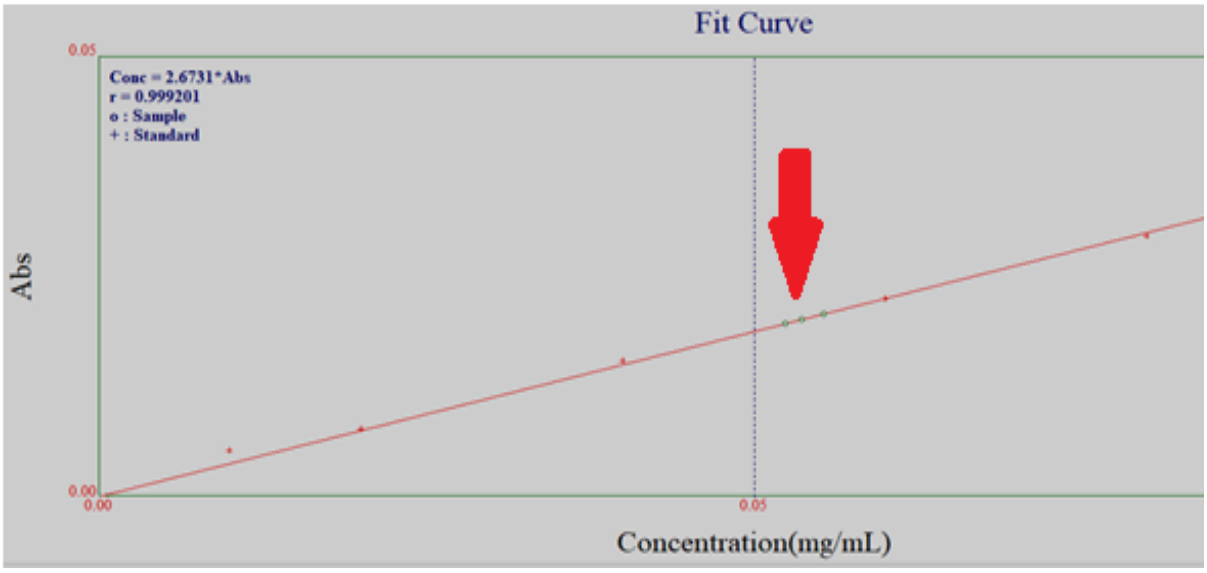

Figure 1 A

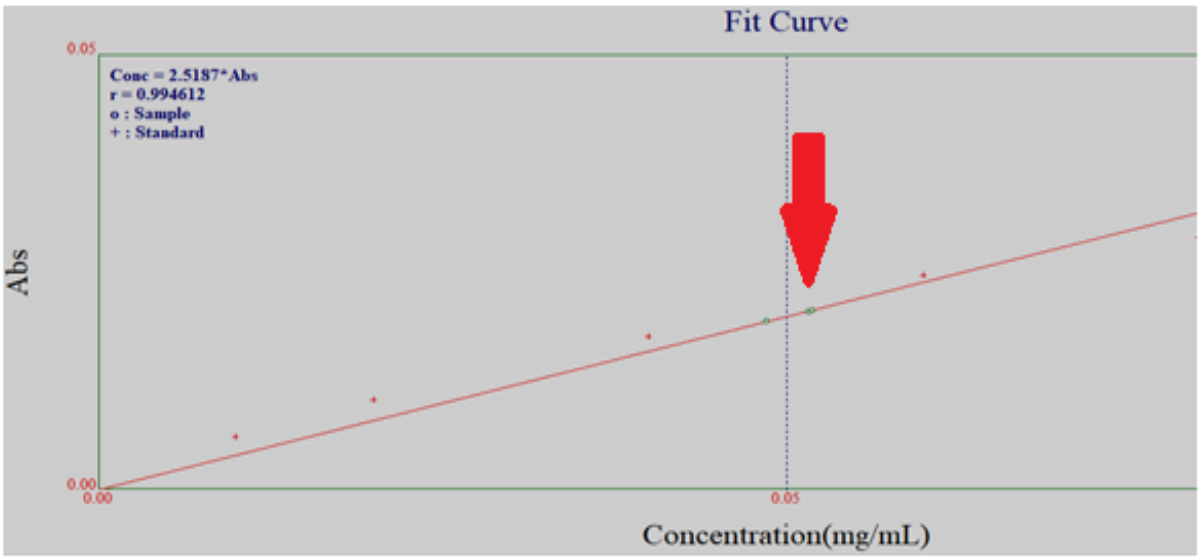

Figure 1 B

The purification of the дeaf protein extract by gel filtration chromatography revealed 4 fractions. Also, 4 fractions were obtained from the extract of *Salvia aethiopis* L. Third and fourth peaks of *Tagetes erecta* L and *Salvia aethiopis* L. were combined and injected in mice (Figure 2 A and B).

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Figure 2 A and B. Chromatogram of fractions obtained by gel filtration chromatography of protein extracts of *Tagetes erecta* L.(A) and *Salvia aethiopis* L. leaves (B).

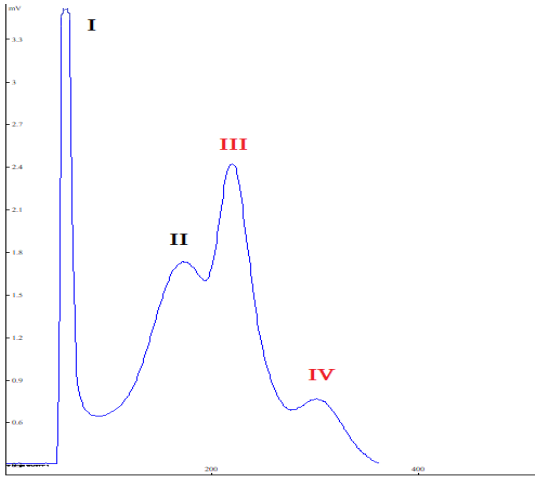

Fig. 2A. Protein fractions of *Tagetes erecta* L. obtained by gel filtration chromatography.

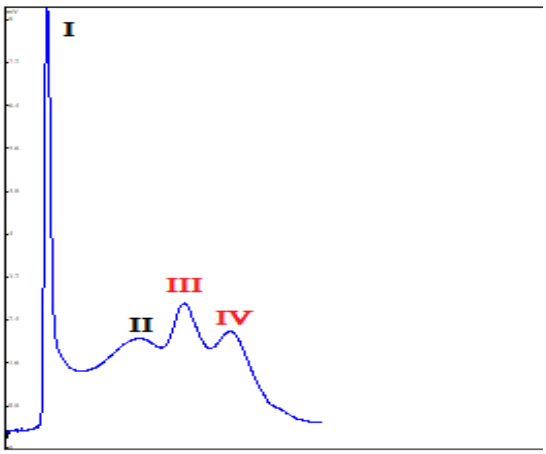

Fig. 2B. Protein fractions of *Salvia aethiopis* L. obtained by gel filtration chromatography

In the next stage of experiments, the third and fourth peaks of *Tagetes erecta* L. and *Salvia aethiopis* L. obtained by gel filtration chromatography were subjected to ion exchange chromatography. The third peak of *Tagetes erecta* L obtained by gel filtration chromatography was separated into three fractions by ion exchange chromatography, and the fourth peak of *Tagetes erecta* L was separated into two fractions (Figure 3).

Figure 3. Ion-exchange chromatography of peaks 3 and 4 of *Tagetes erecta* L. obtained by gel filtration chromatography.

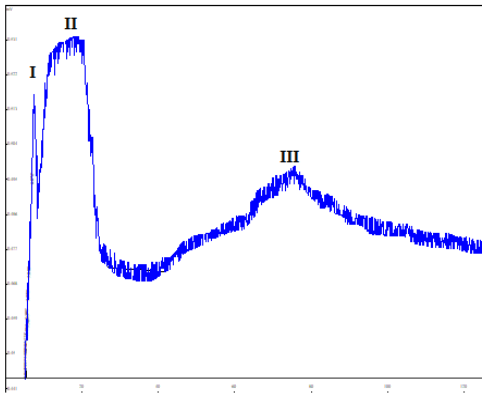

Fig. 3A. The ion-exchange chromatography Of the third by gel filtration chromatography Tagetes erecta L.

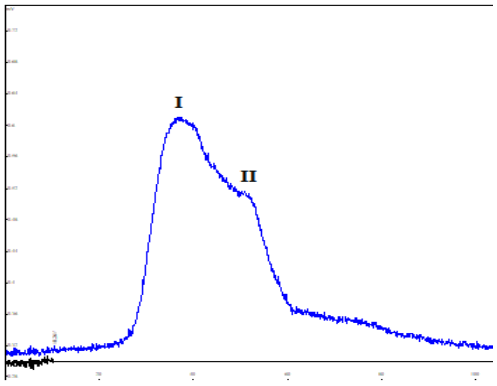

Fig. 3B. The ion-exchange chromatography Of the fourth peak obtained by del filtrtion of chromatography of Tagetes erecta L.

The third peak obtained by gel filtration chromatography from *Salvia aethiopis* L. Leaf extract was separated into two fractions by the ion-exchange chromatography and the fourth peak was separated into 5 fractions (Figure 4).

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Figure 4. Ion-exchange chromatography of peaks 3 and 4 of *Salvia aethiopis* L. obtained by gel filtration chromatography.

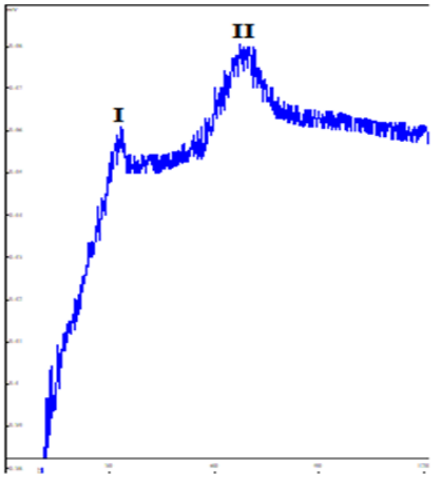


Fig. 4 A. The ion-exchange chromatography of the third peak, obtained by gel filtration chromatography of ***Salvia aethiopis* L**

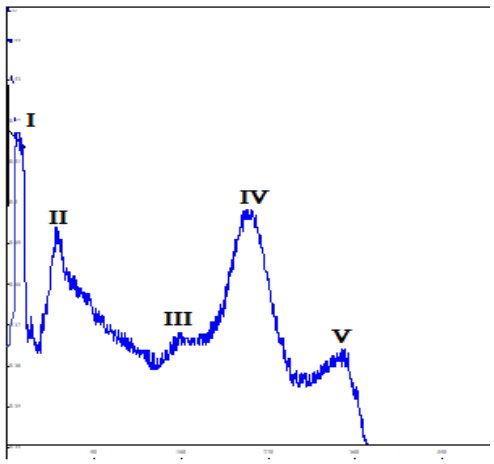


Fig. 4 B.The ion-exchange chromatography of the fourth peak, obtained by gel filtration chromatography **of *Salvia aethiopis* L**

At the next stage of the experiment proteins of *Tagetes erecta* L. and *Salvia aethiopis* L. leaves were subjected to SDS-electrophoresis in 12% polyacrylamide gel.

In both cases, several fractions were identified including proteins with a weight of 23-47 kDa, which most likely act as hepatoprotectors (Figure 5 A and B).

Fig. 5. SDS-PAGE of *Tagetes erecta* L. and *Salvia aethiopis* L. leaf extract in 12% polyacrylamide gel.

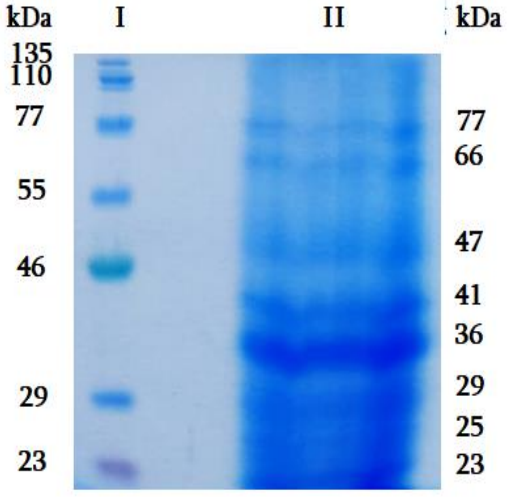


Fig. 5A. SDS-PAGE of ***Tagetes erecta* L**.

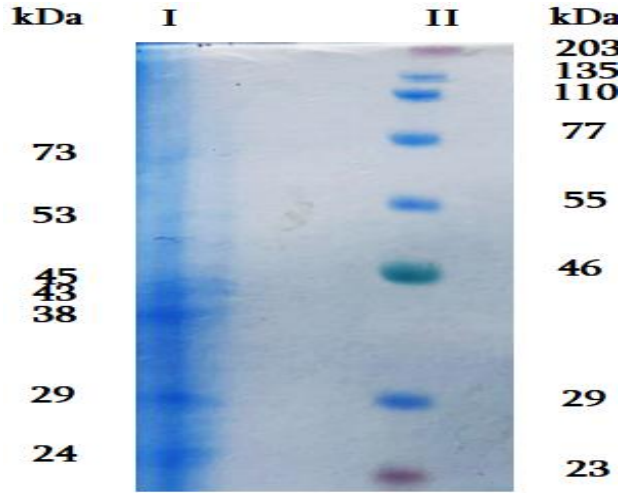


Fig. 5A. SDS-PAGE of ***Salvia aethiopis* L**

A histomorphological pattern of liver tissue showed that intraperitoneal injections of experimental mice with protein fractions obtained from ***Tagetes erecta* L**.

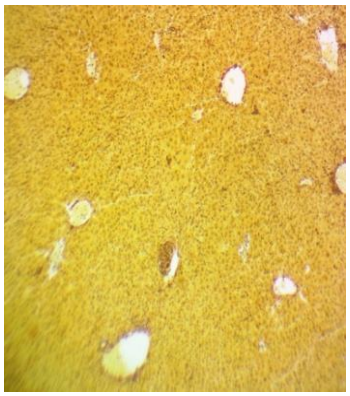
and ***Salvia aethiopis* L**. Leaves during 30 days did not have any pathological effect on the hepatocytes (Figure 6 A, B,C, D,E,F).

DOI: 10.56580/GEOMEDI47

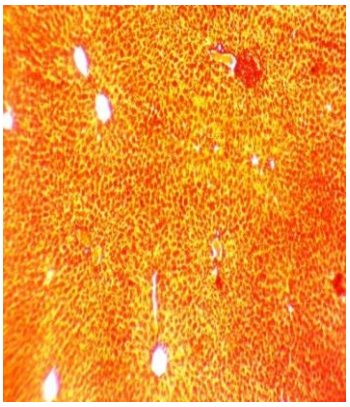
Fig. 6. Effect of 5 mM PBS (A, B, C), *Tagetes erecta* L. and *Salvia aethiopis* L. protein fractions (D,E,F) on mouse hepatocytes.

Fig. 6. A, B, C. Liver tissue of mice injected 30 days with PBS (5mM)

A



B



C

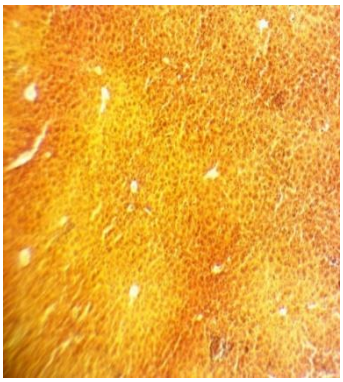
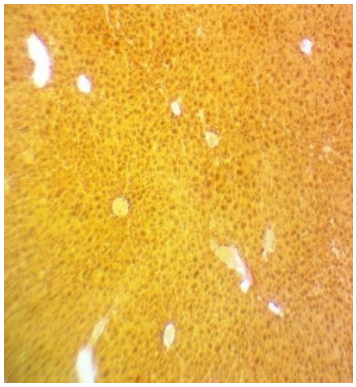
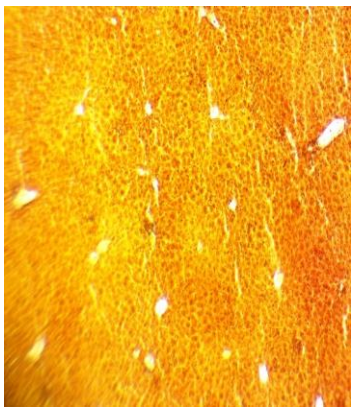


Fig. 6. D, E, F. Liver tissue of mice injected 30 days with *Tagetes erecta* L. and *Salvia aethiopis* L.protein fractions in PBS (5mM)

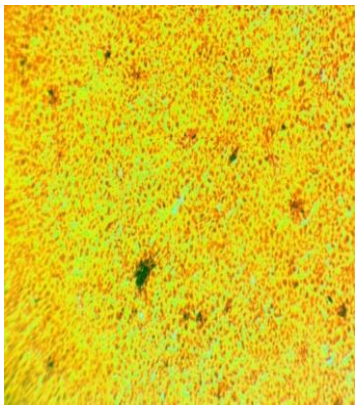
D



E



F



The simultaneous administration of CCl_4 and extract lead to lower development of connective tissue than administration of CCl_4 alone (Figures 7.1-7.2). In the second couple of samples the degree of development of connective tissue was practically the same for administration of CCl_4 and extract and administration of CCl_4 alone (Figures 7.3-7.4).

In the third and fourth samples where CCl_4 And protein fractions were used, the

In the third and fourth samples where CCl_4

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and protein fractions were used, the interlobular connective tissue was visible, although, in CCl_4 and protein fractions variant, a lower degree of damage was observed (Figures 7.5-7.6; 7.7-7.8).

In addition, fuchsinophilic hepatocytes in the samples are less pronounced in joint samples of plant fractions and carbon tetrachloride (10%). It is known from the literature that fucinophilia indicates tissue damage, which was first detected in myocardial cells damaged by coronary heart disease [17].

Therefore, we can conclude that joint injection of *Tagetes erecta* L. and *Salvia aethiopis* L. protein fractions with a concentration of 0.00179 mg/g had weak hepatoprotective action against hepatocyte damage by CCl_4 (Figure 7).

Figure 7. Effect of *Tagetes erecta* L. and *Salvia aethiopis* L. protein fractions on hepatocytes of mice damaged by CCl_4.

The combined effect of CCl_4(10%) and Protein fractions of the extract Tagetes erecta L. and and Salvia aethiopis L on mouse hepatocytes	Damaged mouse hepatocytes 10% tetrachloromethane (CCl_4)

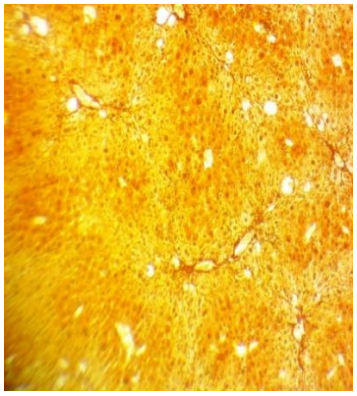

Fig. 7.1. The interlobular borders of the liver are not expressed. Small numbers of numbers of fuchinophilic hepatocytes and steatores are observed in the lobules.

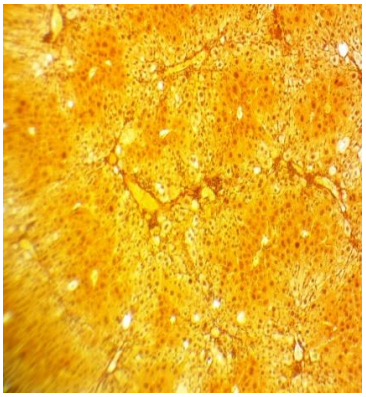

Fig. 7.2. The interlobular borders of the liver are expressed. Fuchinophilic hepatocytes and steatores are observed in the lobules.

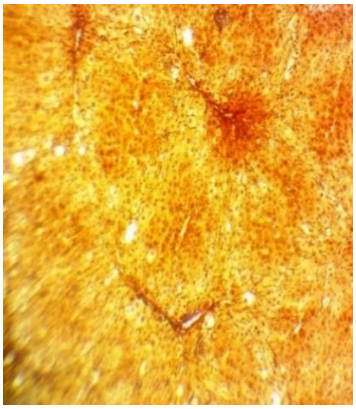

Fig.7.3. Small amount of connective tissue between the liver lobules. Low degree of hepatic steatosis is observed.

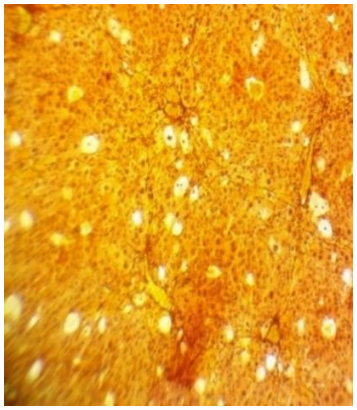

Fig.7.4. Small amount of connective tissue between the liver lobules. More pronounced hepatic steatosis was observed.

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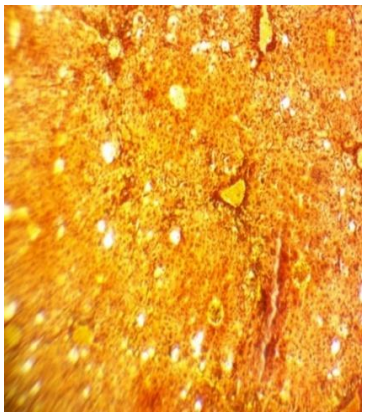

Fig. 7.5. Small amount of connective tissue between the liver lobules. Minor groups of fuchinophilic hepatocytes and steatosis were observed in the lobes.

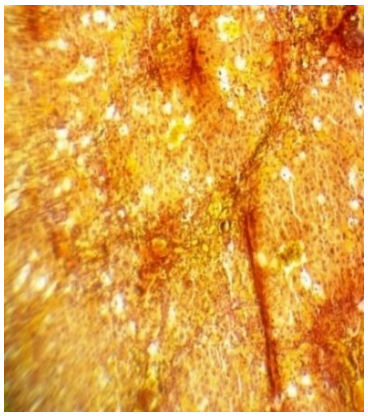

Fig. 7.6. Connective tissue between the liver lobules was expressed. Hepatic steatosis Was observed observed in the liver lobules

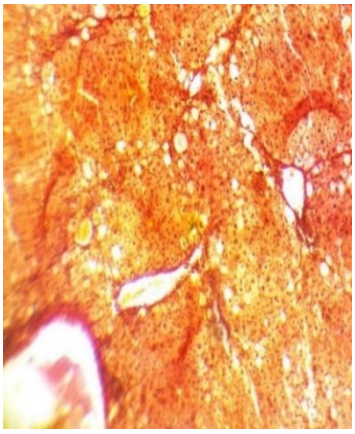

Fig. 7.7. Small amount of connective tissue between the liver lobules. Minor groups of hepatic steatosis Were. Oobserved in the lobules

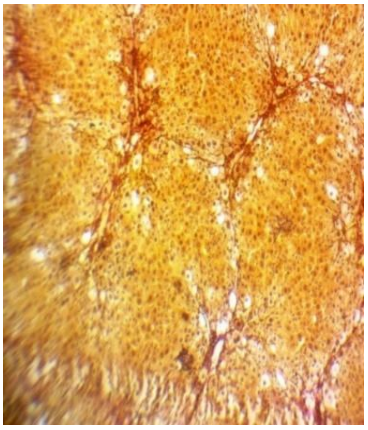

Fig. 7.8. Connective tissue between the liver lobules was well expressed. Hepatic steatosis is observed.

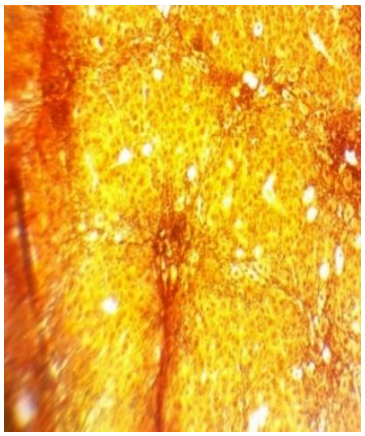

Fig. 7.9. The liver lobules are enlarged. Connective tissue is slightly expressed. Minor groups of fuchinophilic hepatocytes and hepatic steatosis were. Observed in the lobules.

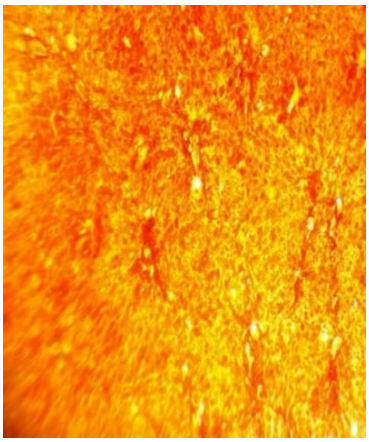

Fig. 7.10. The liver lobules were enlarged. Connective tissue was not expressed. A large number of ofuchinophilic hepatocytes. was observed. Hepatic steatosis was not. Observed in the lobules.

DOI: 10.56580/GEOMEDI47

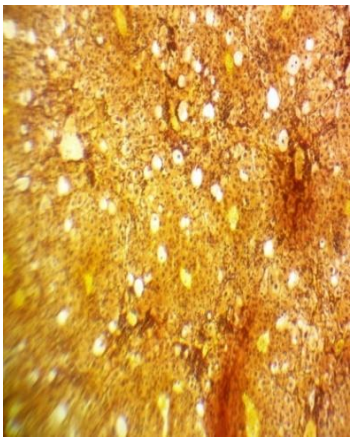

Fig. 7.11. The interlobular borders of the liver with expressed connective tissue. Pronounced hepatic steatosis was observed in the lobules.

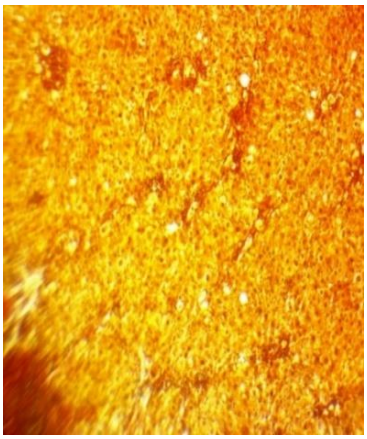

Fig. 7.12. The interlobular borders of the liver with expressed connective tissue. Hepatic steatosis was less expressed.

In addition, in histomorphological images, both in the samples obtained after the treatment with protein fractions and tetrachloromethane and in the samples of the treatments with tetrachloromethane alone, fat clusters were well expressed. Such fat clusters were not observed in the control sample obtained after the treatment with 5% PBS (Figure 6). It is known that tetrachloromethane causes steatosis and fatty dystrophy of the liver [18;19].

In our case, the protein fractions of Tagetes erecta L. And Salvia aethiopis L. reduced the development of steatosis in three of the six samples. In particular, in the second, third and fourth samples. In the first and sixth samples, steatosis is expressed almost identically, both in samples containing an extractive substance and tetrachloromethane (10%), and only in samples exposed to tetrachloromethane (10%). However, steatosis was absent in the fifth sample containing tetrachloromethan. (Fig. 7 – 10).

Conclusions

1. Four protein fractions were obtained from *Tagetes erecta* L. and *Salvia aethiopis* L. by gel filtration chromatography.
2. The obtained fractions were separated by ion-exchange chromatography into several fractions, the third fraction obtained from *Tagetes erecta* L. was separated into three fractions and a fourth fraction of this plant was separated into two fractions. The third fraction of *Salvia aethiopis* L. was separated into two factions, and the fourth fraction was separated into 5 factions.
3. The third fraction of *Tagetes erecta* L. and the fourth fraction of *Salvia aethiopis* L. obtained by gel filtration chromatography at concentrations of 0.0537 mg/ml (by recalculation per animal weight the concentration comprised 0.00179 mg/g) provided weak hepatoprotective effect in mice treated with 10% tetrachloromethane.
4. The third fraction of *Tagetes erecta* L. and the fourth fraction of *Salvia aethiopis* L. obtained by gel filtration chromatography at concentrations of 0.0537 mg/ml (by recalculation per animal weight the concentration comprised 0.00179 mg/g) reduced the development of hepatic steatosis in three of the six samples.
5. The SDS-PAGE of *Tagetes erecta* L. and

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Salvia aethiopis L. proteins in a 12% gel revealed several fractions, including proteins with a molecular weight of 23-47 kDa, which are most likely involved in protecting hepatocytes damaged from 10% tetrachloromethane.

მცენარეების *Tagetes erecta L.* და *Salvia aethiopis* L. ფოთლების გელ-ფილტრაციული ქრომატოგრაფიით მიღებული ცილოვანი ფრაქციების ჰეპატოპროტექტული თვისებების შესწავლა თაგვების ტეტრაქლორმეთანით (CCl_4) დაზიანებულ ჰეპატოციტებზე

მარინა ფირცხალავა[1], მალხაზ ვახანია[1,2*], თეიმურაზ თავხელიძე[2], მარინა გოგოძე[2], მარიამ კობიაშვილი[2], თორნიკე მინდიაშვილი[2].

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აბსტრაქტი

სტატიაში მოცემულია მცენარეების *Tagetes erecta L.* და *Salvia aethiopis L*-ს ცილოვანი ექსტრაქტების გელ-ფილტრაციული ქრომატოგრაფიის მეთოდით დაყოფილი ფრაქციების მოქმედება 10%-იანი ტეტრაქლორმეთანით დაზიანებულ თაგვების ჰეპატოციტებზე. აღნიშნული მცენარეების მესამე და მეოთხე პიკების ცილოვან ფრაქციებს გააჩნიათ სუსტი ჰეპატოპროტექტული თვისებები. თითოეული პიკი იონცვლადი ქრომატოგრაფიის მეთოდით ასევე იყოფა რამდენიმე ფრაქციად.

SDS-გელ-ელექტროფორეზის მიხედვით გამოვლინდა ფრაქციები, მათ შორის მოლეკულური მასით 23-47 kDa, რომლებიც დიდი ალბათობით უნდა მონაწილეობდნენ ჰეპატოციტების დაცვაში.

საკვანძო სიტყვები: *Tagetes erecta* L., Salvia aethiopis L., იმერული ზაფრანა, სალბი, ცილები, გელ-ფილტრაციული ქრომატოგრაფია, იონცვლადი ქრომატოგრაფია, ტეტრაქლორმეთანი CCl_4, გელ-ელექტროფორეზი, ჰისტომორფოლოგია, ჰეპატოციტები.

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