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Applicability of alkanet (*Alkanna tinctoria*) extract for the histological staining of liver tissue

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ABSTRACT

Staining of tissue is a significant process in histotechnology and staining techniques are used in the examination and diagnosis of diseases. The increase of international awareness for the environment, ecology, and health directed people to reduce toxic effluents and stop usage of dangerous chemical dyes. The present trend throughout the world is shifting towards to use of natural products over their synthetic alternatives. Therefore, in this study, the dyeing ability of alkanet plant on the cells of unstained sections taken from Wistar rat liver was investigated. The extract of *Alkanna tinctoria* contain naphthaquinone group of dyestuff. Chemical characterization of *A. tinctoria* extract was performed with HPLC-TOF/MS and UV spectra analysis, respectively. Different metal salts were used in the staining processes in order to obtain different colors. In the staining of without adding mordant (metal salt) to the extract, pale brown staining was obtained in the cytoplasm. Light pink color was obtained with the addition of $CuSO_4$ mordant. Dark pink-red color was achieved by adding alum, and also $NiSO_4$ was added for light purple staining. The staining of cytoplasm of different cells in liver tissue was achieved successfully with the extract of *A. tinctoria* and different colors were obtained using various mordants.

1. Introduction

Natural dyes are obtained from plants, animals, and minerals and have long been used in the dyeing of textiles, foods, cosmetics, pharmaceuticals etc. Plant based natural dyes have a wide range of color shades and can be easily obtained from the different parts of the plant. Although hematoxylin is the most commonly used dye in histology, it has the disadvantages of high cost and supplement problems, as well [1,2]. In recent years, usage of natural dyes in the staining of various cells has gained interest [3] due to their eco-friendly, biodegradable, and less toxic properties [4]. There are several studies [2,5–9] related with the usage of natural dyes for staining processes including histochemistry, histology, and histopathology [10]. Staining of cells is a useful process especially for clinical diagnosis of some diseases [11].

Alkanna tinctoria (alkanet) that belongs to the Boraginaceae family is widely spread in Southern Anatolia and Mediterranean [12]. Its roots are used for the treatment of wounds, ulcers, inflammation, and fever [13,14] and contain alkannin and shikonin (Fig. 1) compounds which are responsible from the colorization [15,16]. These compounds are used as natural sources of red pigment in cosmetic, textile, and food industry, as well [13,17].

Generally, metal mordants are used in the dyeing processes in order to enhance the color strength of the dyed material and improve the staining ability of the dye, vice versa [18]. Mordants provide an affinity between the tissue and the dyestuff of the plant by forming a coordination complex via covalent bonding [19,20]. A metal salt plays an important role in the improvement of the staining of not only cartilage but also nuclei [21].

This study was therefore undertaken to emphasize the importance of using mordants to achieve purple and pink colors in tissue staining since there is no vivid, and good color staining on the cell cytoplasm with alkanet extract. In addition, alkanet extract was characterized chemically by HPLC-TOF/MS and UV spectra in order to determine the coloring agents which are responsible from the stained tissues.

2. Material and methods

2.1. Ethical statement

The experiments on rats were carried out according to the ethics of scientific research, as the approval of the Ethnical Scientific Committee for Animal Rights was obtained under No. (16/144).

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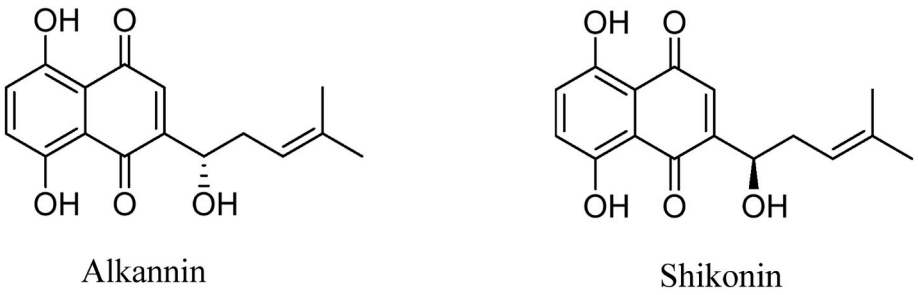


Fig. 1. Chemical structures of alkannin and shikonin.

2.2. Materials

Dried alkanet roots were purchased from a local producer in Turkey. All chemicals were of analytical grade and supplied from Sigma-Aldrich (USA) and Merck (Germany), respectively. HPLC analysis was carried out with an Agilent 6210 HPLC TOF-MS instrument. UV spectra was recorded on a PerkinElmer 200 Series Spectrophotometer.

2.3. Preparation of animals

Two rats (male/female) were used with a weight ranging between 150 and 250 g. The rats were raised in wire cages and the appropriate food and drink were placed, with proper temperature, light and humidity controlled [3].

2.4. Preparation of sections

A tissue sample from the Wistar rat liver organ was fixed in 4% formalin. Tissue was embedded in paraffin, after doing tissue following process with different solvents and obtained 5 μm thickness paraffin sections by a microtome (Mslk 221). The preparates containing sections were ready for deparaffinization [22].

2.5. Tissue handling with deparaffinization

Three changes of xylene are usually sufficient for the removal of the paraffin on the tissue. Xylene I – III solutions were prepared in order to remove the paraffin completely. The tissue preparates were placed into baths of xylene I and II for a minimum of 10 min and into that of xylene III for a minimum of 5 min, as well. After the xylene III bath, the tissue preparates were placed into ethanol to remove the xylene. The ethanol concentrations were gradually reduced from 100% to 95% and then to 70%. The tissues were placed into each ethanol bath for 5 – 10 min. Finally, the preparates were rinsed with running tap water to remove the ethanol [23].

2.6. Extraction of Alkanna tinctoria

The dried roots of A. tinctoria were powdered using a mixer. The plant powder (20 g) was extracted with 200 mL of ethanol solution (70%) at 37°C for 24 h. Impurities were removed by filtering the mixture with Whatman no 1 paper, and this step was repeated twice to ensure the clarity of the extract. The solution was vaporized in a water-bath until the final volume was 100 mL to get more intense dye solution. The process was repeated twice in order to obtain 200 mL of dye solution. The concentrating of the dye powder in solution was 14% and pH was 6.5. The resulting liquid was kept at 4°C for use in tissue staining used in the research [24].

2.7. Chemical characterization of A. tinctoria extract

Chemical characterization of alkanet extract was performed on HPLC TOF-MS instrument with a ZORBAX SB-C18 column. The conditions of the chromatographic analysis was as follows: injection volume: 10 mL; column size: 4.6 × 100 mm, 3.5 μm. Hexane:2-propanol (90:10) was used for the mobile phase with a flow rate of 0.6 mL/min and the temperature was set at 35°C. TOF analyses were carried out in positive ion mode; gas temperature, 325°C; drying gas flow, 10 mL/min; fragmentor voltage, 175 V.

2.8. Dyeing procedure with Alkanna tinctoria

Alkanet extract (200 mL) was divided into 4 parts, one of which was left as blank, and 3 g of $KAl(SO_4)_2{\cdot}12H_2O$ (alum), $CuSO_4$ (copper), and $NiSO_4$ mordants were added to the 50 mL of the solution, separately. Then, the liver tissue was put into these solutions and they were left to incubation at 60°C, for 60 min. Afterward, they were washed with distilled water and dipped in different concentrations of ethyl alcohol for 180 s. Images of the stained tissues were screened under the oily lens of the optical microscope [3]. Different colors were stained in the cytoplasm of epithelial cells in liver tissue in the presence of alkanet extract with various mordant substances. The results were compared with H&E stained sections (control) which were studied by Mortazavi

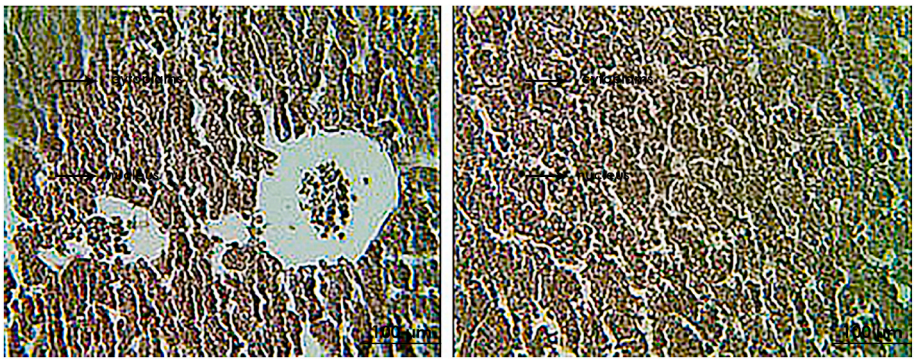


Fig. 2. Liver tissue section stained without mordant.

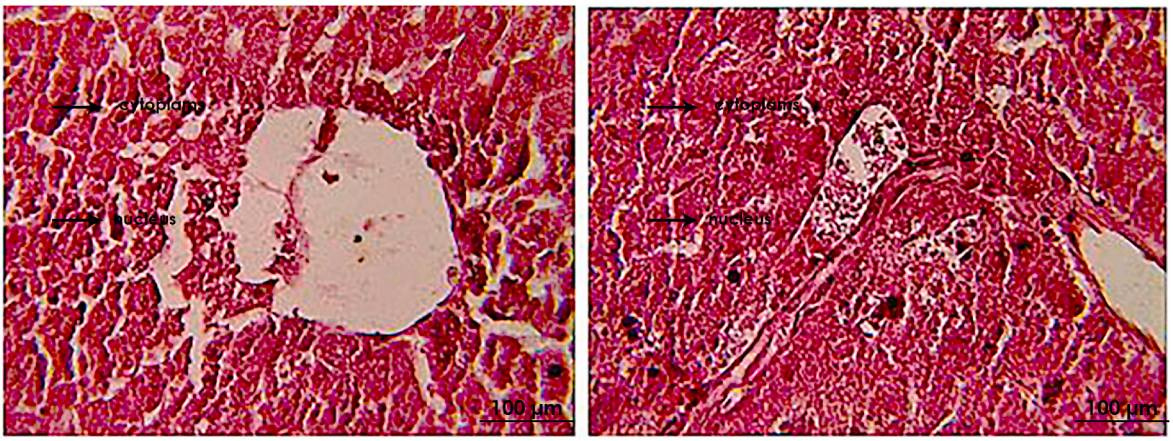


Fig. 3. Liver tissue section stained with $CuSO_4$ mordant.

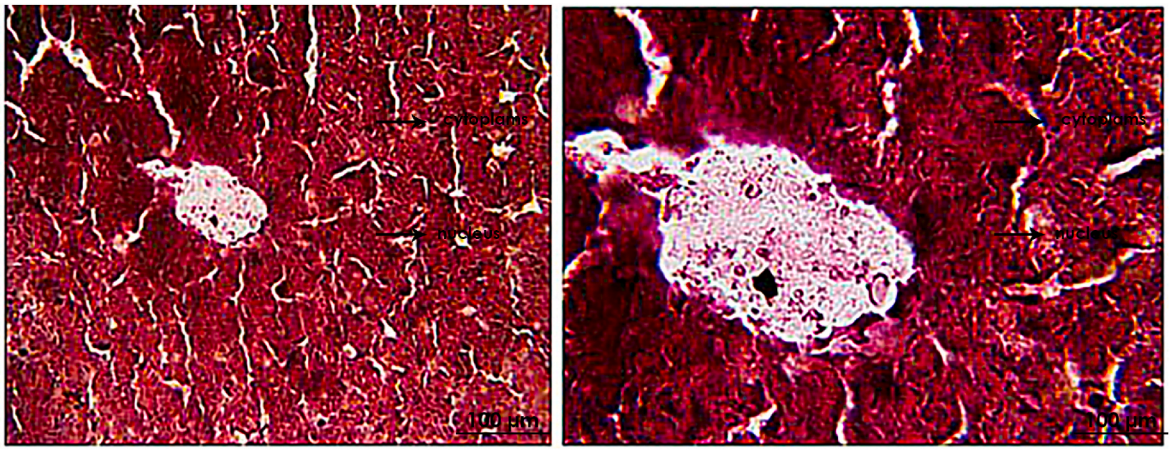


Fig. 4. Liver tissue section stained with alum mordant.

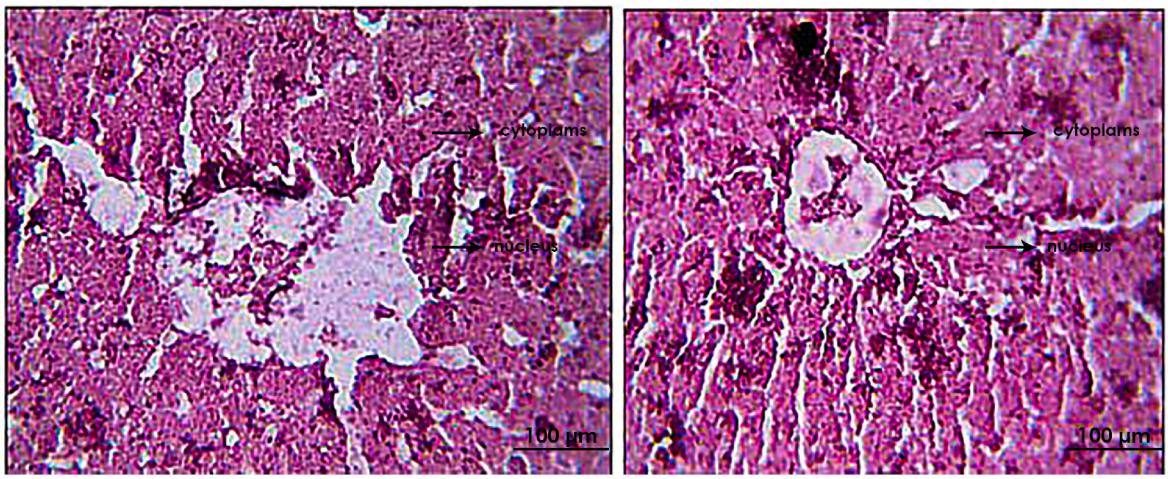


Fig. 5. Liver tissue section stained with $NiSO_4$ mordant.

[22].

3. Results and discussion

Photographs of the liver tissue that stained with the extract of alkanet were taken under a light microscope. In the staining of without adding mordant to the extract (pH 6.5), pale brown staining was obtained in the cytoplasm (Fig. 2). Light pink color was obtained with the addition of $CuSO_4$ mordant (Fig. 3). Dark pink-red coloring was achieved by adding alum (Fig. 4), and also $NiSO_4$ was added for light purple staining (Fig. 5). Liver tissue stained with the hematoxylin-eosin dye duo used routinely in medical laboratories takes place in Fig. 6 for positive control. Here, the cell nuclei in the liver tissue are colored purple and the cytoplasm pink-red.

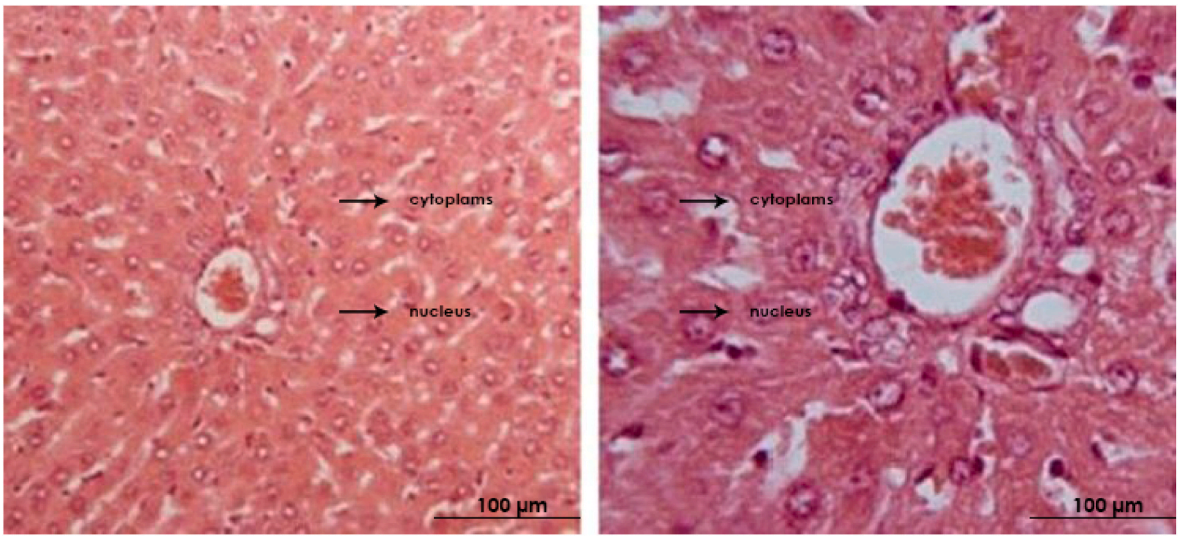


Fig. 6. Photographs of dyed liver tissue with Hematoxylin-eosin (Mortazavi et al., 2016).

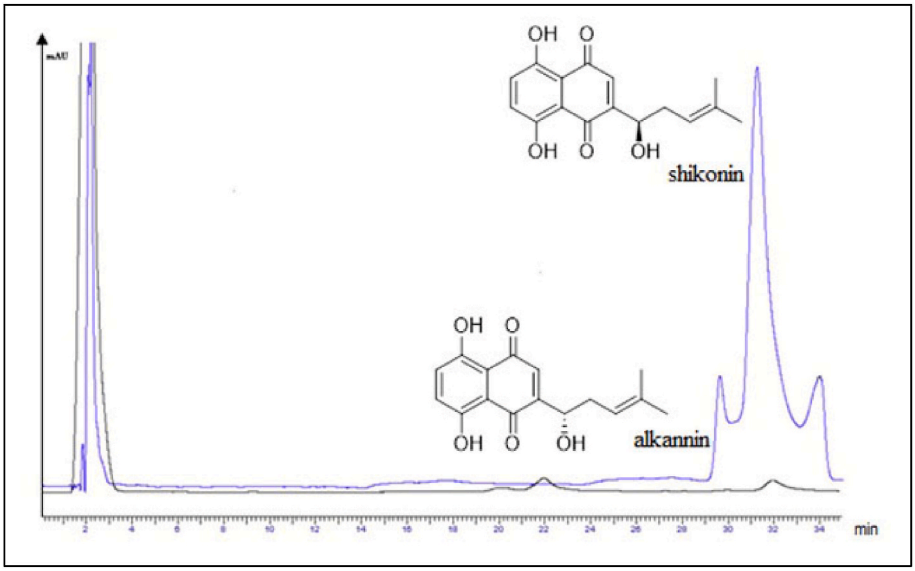


Fig. 7. HPLC chromatogram of alkanet extract.

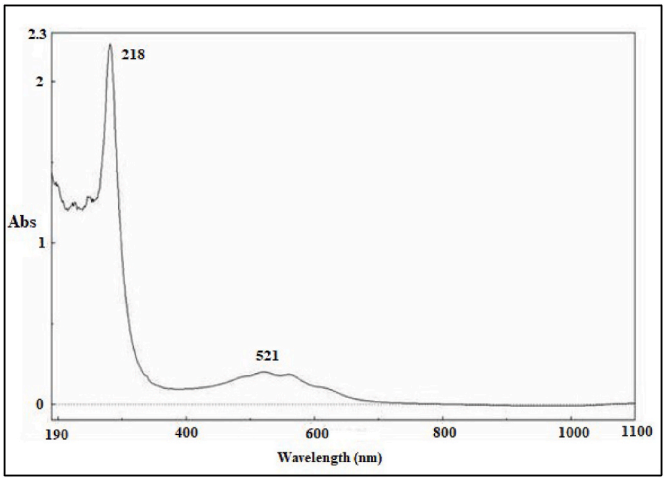


Fig. 8. UV spectra of alkanet extract.

The major compounds (alkannin and shikonin) of alkanet were confirmed from the HPLC analysis (Fig. 7). Alkannin and shikonin compounds are enantiomer of each other; i.e. they present as rasemic mixture in the extract. They exist as mainly monomeric A/S derivatives [25] in alkanet extract. It is observed that amount of shikonin is higher than alkannin from the HPLC chromatogram (Fig. 7) of the extract. The UV spectra of the extract (Fig. 8) is in accordance with previous studies [26,27] also confirm that the major compound of the alkanet extract is shikonin. Therefore, proposed dyeing mechanism (Fig. 9) is explained over shikonin (dye)-mordant-cytoplasm (amino acid) coordination complex.

Alkanet extract can act as an indicator due to its different colors in various pH values [28]. The pH of the extracts became more acidic in case of alum (pH 3.8), $NiSO_4$ (pH 4.1), and copper (pH 4.4) mordants, respectively. It is worth mentioning that, pH plays an important role in the formation of the final color in the staining processes.

In a previous study, the staining of the cytoplasm of rat liver tissue was performed with Punica granatum flower extract [3]. However, there are no tissue studies previously stained with the extract of the *A. tinctoria* plant. Also, staining with different metal salts was done with

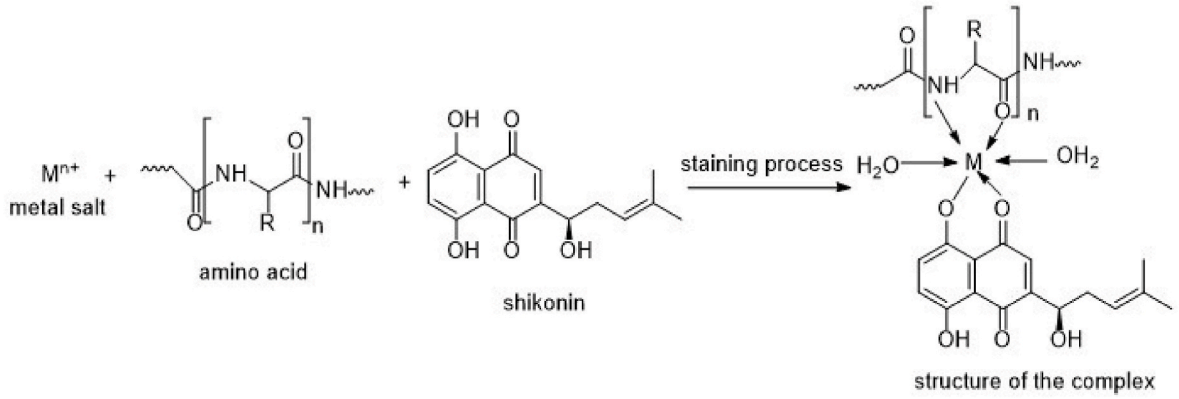


Fig. 9. Staining mechanism of cytoplasm with alkanet extract in the presence of metal mordant.

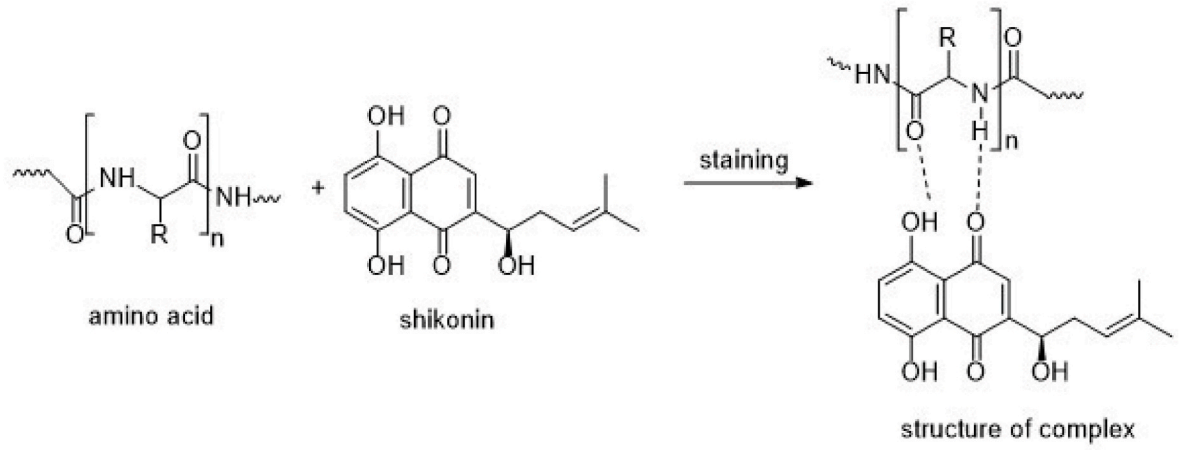


Fig. 10. Staining mechanism of cytoplasm with alkanet extract without mordant.

hematoxylin dye. Cell staining in different colors was performed with solutions of hematoxylin, which are widely used in nucleus staining, containing iron and alum metal salts. Early studies revealed that, dye-metal complex prefer to bind arginine-rich proteins rather than DNA [29–31].

Preliminary phytochemical analysis of A. tinctoria roots revealed that it contains alkannin, shikonin, naphtharzin, shikalkin, acetylalkannin, arnebifuranone, alkanfuranol, 5-methoxyangenylalkannin, alkandiol, acetylshikonin, angelyalkannin, and dimethylacryl alkannin [17,32,33]. The major enantiomeric naphtoquinones alkannin and shikonin exhibit antitumor activity [34–38]. It is known that, 1,4-naphthoquinones, alkannin, and shikonin compounds have the ability of undergoing reduction and cyclic oxidation which generates ROS [39–41].

Both alkannin and shikonin contain hydroxyl (-OH) and carbonyl (C═O) groups that can form strong chemical interactions between the metal cation and amino (-NH_2) and –OH groups of protein via coordinate covalent bonding. In the presence of metal salt, a more stable complex takes place. The interaction between protein and metal ion results unoccupied coordination sites. These sites may be occupied with the functional groups of the protein such as amino and carboxyclic groups [42]. Thus, such coordination tendency could change the color of the stained tissue. The colored complex may occur with the interaction between the functional groups of the dye and protein in the staining of the tissue without mordant. Hence, the non-mordanted staining of the liver tissue was proposed as in Fig. 10.

4. Conclusion

In this study, the necessity of using mordant in cell staining with *A. tinctoria* was revealed, since there is brown staining in the cytoplasm without mordant and more vivid dyeing with other mordants. Purple and pink staining were obtained in the presence of $CuSO_4$ and $NiSO_4$ mordants, respectively. Alkannin and shikonnin dyestuffs were detected in the *A. tinctoria* extract with HPLC analysis which are responsible from the staining of the tissues.

Further studies can be conducted on the staining performance of alkanet on different cells. The staining ability of alkanet on cell elements may examine by electron microscopy. Since the color substances in the extract adsorb to the cell surface (via coordinate covalent bonding) in the staining process, it will be easy to clarify their attachment on the surface with electron microscopy and even with which cell element.

In conclusion, alkanet extract can be used in the staining of liver cells to make evaluations such as distinguishing normal and abnormal liver cells in clinical laboratory studies.

Declaration of competing interest

The authors declared that there is no conflict of interest.

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