

Dual Staining Effect of Extracts Roselle (*Hibiscus Sabdariffa* L.) and Red Cabbage (*Brassica Oleracea*) on Wistar Rat Tissues

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Research Article

Keywords: Hibiscus sabdariffa, Hematoxylin-eosin, Wistar albino, Brassica oleracea, Kidney, Liver

Posted Date: February 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-214239/v1>

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Abstract

Background: Apart from the areas where natural dyes are used a lot, if staining studies on tissues increase, their use in pathology laboratory will become widespread. Two basic elements in cell tissues, the nucleus, and cytoplasm, should be stained and evaluated together for histopathological diagnosis. The most commonly used dye combinations for this diagnosis are Hematoxylin-Eosin dyes, also known as nuclei and cytoplasm dyes. Histological staining studies of natural origin did not reveal staining studies on the same tissue with the extracts of two plants.

Methods and Results: In this study, roselle (*Hibiscus sabdariffa* L.) and red cabbage (*Brassica oleracea*) leaves were used as a source of natural dye and also liver and kidney tissues of Wistar albino rat as dye biomaterials. The nuclei and cytoplasm regions of rat kidney and liver tissues were stained in different colors with these plants and the use of metal salts. Relying upon hematoxylin-eosin should it ever be scarce will result in the scarcity of plant-based dyes on the market.

Conclusion: Since a more colorful, different nucleus and cytoplasm staining was obtained compared to previous work with the same tissues. It is expected that the introduction of new natural dye alternatives where histological dye production is not widespread will contribute to the world's economy and industry.

Introduction

Natural dyes, generally supposed to be a cheap, non-toxic, renewable and sustainable resource with minimal environmental impact, have attracted the attention of the scientific community to use them in a variety of traditional and newly discovered application disciplines [1]. It is known that natural dyes have been used for coloring Textil materials [2]. However, there are also studies with natural dyes on the staining of tissues, which are a different discipline [3]. Biopsy specimens or tissue fragments are taken from animals or humans in medical and research laboratories. These are kept and processed with many chemicals in short periods in the pathology laboratory. The purpose of this process called tissue follow-up is to clean and polish the sections to be taken from the tissue samples and make them suitable for examination and staining. Tissue tracking consists of three basic stages. Step 1 is the dehydration, which is the removal of water from the tissues. During the process, the tissues are kept in stepwise ethyl alcohol solutions from 70 % to absolute alcohol. In Step 2, the water is removed but the alcohol remains in the tissue. Tissues are held in toluene and chloroform, organic apolar solvents. In the third and final step, the rigidity of the tissue is necessary for subsequent thin sectioning. For this purpose, melted paraffin tissues are left in cassettes. When the paraffin has hardened the tissue is ready for a microtome to cut through the paraffin. Tissue sections with paraffin are transferred to slides. Before using chemical and dye solutions, the paraffin must be dissolved [4, 5].

The Hematoxylin "log-tree" (*Haematoxylum campechianum*) or "heart tree" is a Mexican-derived plant. The bark can be processed with a mix of hot water and urea and to obtain the dye called hematoxylin. Hematoxylin is used in cell staining, after oxidation, it can be used in cell staining. It is available under

different names since the durability of dye obtained when exposed to natural chemical oxidation and the color shades in the tissue are different. Metal salts known as mordant, alum, iron sulfate, tungsten, molybdenum, and lead salts can be used since the oxidized dye cannot stain the tissue alone. In particular, nucleus dyeing does not take place without iron sulfate [6–8].

Each medicinal laboratory has its own hematoxylin-eosin (HE) dual staining procedure that starts with xylene. For example, the HE dual staining method routinely used in Ankara University. This method is applied similarly in many laboratories around the World [9, 10]. Studies using water and ethanol extracts of *Hibiscus sabdariffa* in tissue dyeing are more numerous than dyeing with red cabbage leaves (RC). In these studies, *Hibiscus sabdariffa* L. (HS) extract and pink-light red staining were performed in the cytoplasm [11, 12]. Red cabbage leaves extract was used as a drug dye [13]. In addition to tissue staining, there are many studies in the literature regarding the determination of biological activities of plant extracts [14–17].

In this study, based on the hematoxylin-eosin dual staining method, which is used routinely, studies were performed using alum and ferrous salts in red cabbage extract instead of hematoxylin and alum salt in *Hibiscus sabdariffa* extract instead of eosin. The colors given by hematoxylin and eosin in the nucleus and cytoplasm were taken as examples. An environmentally friendly, natural sourced dye combination was created.

Materials And Methods

Hibiscus sabdariffa L and red cabbage (*Brassica oleracea*) leaves were obtained from the local markets of Kayseri. Preparing of two plant extracts was taken place like below. All the chemicals were used in analytical pure. Alum ($KAl SO_4 \cdot 12H_2O$) was used 5-10g/ in 50ml extract. $FeSO_4 \cdot 5H_2O$ was used 10–20 g/ in 50ml extract. DDW water.

Tissue Supply

In this study, 1 Wistar albino female rat, weighing 150–250 g and raised at the Erciyes University Experimental and Clinical Research Center (DEKAM) was used. This study was approved by the Erciyes University ethics committee dated 16 November 2016 and numbered 16/144. The rat was kept in cages, at 21°C with normal daily organization, 12 h of light/dark, and was fed and watered appropriately. The rat was put to euthanized with ketamine + xylazine and its tissues were taken. All of the procedures were performed according to ethical guidelines [3].

Preparation of *Hibiscus sabdariffa* L. Extract

The dried of *Hibiscus sabdariffa* L. was ground to a dark red-black powder using a manual grinding machine (Waring, commercial). 50 g of the dry powder of the plant was weighed (Schimadzu bl 3200). The powder was put in 100mL chloroform, boiled for five minutes. Distilled water was added to the

remained solution. This dye solution was used directly or stored in a refrigerator at 4°C and then was used for staining.

Preparation of Red Cabbage Leaves Extract

Red cabbage leaves were ground to a dark red-black powder with the use of a manual grinding machine (Waring, commercial). The dry powder of the plant was weighed at 50 g (Schimadzu bl 3200). The powder was left in 100mL water for 24 hours at room temperature. Then the extract (50 % w/v) was filtered two times with Whatman No.1 filter paper. The Filtrate was boiled until half of the water had evaporated. The remaining blue-purple colored filtrate was used as the dye solution. The filtrate was stored in the refrigerator at 4°C and then was used for staining [12, 3].

Preparation of the Tissue Preparates

The tissue samples taken from the rats were fixed in a 4% neutral buffered formaldehyde solution. After fixing, routine histological tissue follow-up steps were performed. The tissue samples were first dehydrated through an increasing series of ethyl alcohol made transparent with xylene, and then blocked by burial in paraffin. The 5 µm cross-sections taken from the tissue paraffin blocks for dyeing were then applied the staining procedure to see the effect of the *Hibiscus sabdariffa L.* and red cabbage leaves. The intensity of staining was evaluated as negative (-), weak (+), medium (++) , and very intense (+++).³ The best staining intensity (+++) in the evaluation of the staining result was considered as H&E stained tissue. Others have been evaluated accordingly.

Deparaffinization of Tissue Preparation

Paraffin was removed from the preparete by the classical method. Preparates were left in an oven at 60°C for 30 minutes. They were removed from the oven and allowed to cool to room temperature for 3–5 min and put them into the xylene [8].

Hematoxylin-Eosin (H&E) Staining

The dyeing process was respectively applied to the deparaffinized kidney and liver preparates as below [7, 8, 10].

1. Immersed in Xylene for 3 min, and repeated three times
2. Immersed in E. Alcohol for 3 min, and repeated three times (from 70% Ethyl Alcohol to 96 %)
3. Washed with distilled water for 3min.
4. Immersed in Hematoxylin solution for 6- 8min
5. Washed with distilled water for 3 min
6. Immersed in 1% Ammonia water for 2 min
7. Washed with distilled water for 3 min
8. Immersed in Eosin solution for 10 -12min
9. Washed with distilled water for 3 min
10. Immersed in E. Alcohol for 3 min, and repeated three times (from 96% Ethyl alcohol to 70%).

11. Immersed in Xylene for 3 min, and repeated three times
12. Preparates closed with entellan

Photographs of the tissues on the slides are shown in 100× magnification by light microscopy (Olympus BX-51, Japan).

Natural Staining with extracts

Hibiscus sabdariffa and red cabbage leaves extract with and without metal salt instead of hematoxylin-eosin were used in the H&E (control) routine dyeing process. Below processes were separately done to the preparates containing liver and kidney tissues.

Staining Process 1

1. Immersed in Xylene for 3 min, and repeated three times
2. Immersed in E. Alcohol for 3 min, repeated three times
3. Washed with distilled water for 3min.
4. Immersed in Red cabbage (RC) extract for 6-8min
5. Washed with distilled water for 3 min
6. Immersed in 1% Ammonia water for 2 min
7. Washed with distilled water for 3 min
8. Immersed in *Hibiscus sabdariffa* (HS) extract for 10-12min
9. Washed with distilled water for 3 min
10. Immersed in E. Alcohol for 3 min, and repeated three times
11. Immersed in Xylene for 3 min, and repeated three times
12. Preparates closed with entellan

Staining Process 2

1. Immersed in Xylene for 3 min, and repeated three times
2. Immersed in E. Alcohol for 3 min, and repeated three times
3. Washed with distilled water for 3min.
4. Immersed in Red cabbage (RC) extract + FeSO_4 + Alum for 6-8min
5. Washed with distilled water for 3 min
6. Immersed in 1% Ammonia water 2 min with distilled water for 3 min
7. Immersed in *Hibiscus sabdariffa* (HS) extract + Alum for 10-12min
8. Washed with distilled water for 3 min
9. Immersed in E. Alcohol for 3 min, and repeated three times
10. Immersed in Xylene for 3 min, and repeated three times
11. Preparates closed with entellan

Photographs of the tissues on the slides are seen in 100×, 200× magnification by light microscopy (Olympus BX-51, Japan).

Phytochemical Analysis of extracts

The phytochemical analysis steps below were applied separately to the extract of *Red cabbage* obtained in water and the chloroform extract of *Hibiscus sabdariffa* [18].

Test for Saponin. 2 ml extract was placed in a test tube and shaken vigorously. The formation of stable foam was taken as an indication of the presence of saponin.

Test for Phenol and Tannin, 2 ml extract was mixed with 2 ml of 2 % solution of ferric chloride (FeCl₃). A blue-green or black coloration indicated the presence of phenol and tannin.

Test for Terpenoid (Salkowski's Test), 2 ml extract was mixed with 2 ml of chloroform. Then 2 ml of concentrated sulphuric acid was added carefully and shaken gently. A reddish-brown coloration of the interphase was formed to show positive results for the presence of terpenoid.

Test for Flavonoid (Zinc-Hydrochloride reduction test), 2 ml extract was mixed with zinc dust and concentrated hydrochloric acid was added to it dropwise. It gave red color after a few minutes indicating the presence of flavonoid.

Test for Glycoside, 2 ml extract was mixed with 2 ml of glacial acetic acid containing 2 drops of 2 % FeCl₃. The mixture was poured into another tube containing 2 ml of concentrated sulphuric acid. A brown ring at the interphase indicated the presence of glycoside [18].

TLC analysis of extracts

The extracts of *Hibiscus sabdariffa* and red cabbage were subjected to thin layer chromatography solvent system. The fractionation patterns of the extract produced by different solvent mixtures. But, Ethyl acetate: Hexane (1:4) as optimal solvent produced sharp bands with good resolution.

Results

Table 1. Results of dyeing on kidney and liver tissues by staining processes

Process Number	The intensity of dyeing on tissue		Colors of cell elements	
	Cytoplasm	Nucleus	Cytoplasm	Nucleus
1	-	-	Very pale	Very pale
2	++	++	Red-pink	Red-pink

Red-pink medium intensity (++) staining was performed with the *Hibiscus sabdariffa* instead of eosin and also medium (++) purple-colored staining was done with red cabbage instead of hematoxylin in the nucleus. Microscopic examination results were shown in Table 1.

The extracts of basil and alkanet plants instead of red cabbage were used for nucleus staining since they were purple-blue in color, but they did not give positive results (-) in staining. The cell cytoplasm was stained by chloroform extract of the *Hibiscus sabdariffa* L. When the extracts of the *Hibiscus sabdariffa* and red cabbage were applied to the tissues without using mordant, no positive results (-) were obtained (see Dyeing process 1). Photographs of negative tissue staining studies are not included in this study. But positive results were shown in Fig. 1 and Fig. 2.

Table 2. Phytochemical analysis results of plant extracts

Extract	Phenols & tannin	Saponin	Terpenoid	Glycoside	Flavonoid
<i>H. sabdariffa</i>	+	-	+	-	+
Red cabbage	-	-	-	-	+

Note: positive (+): the presence of constituent; negative (-): the absence of constituent.

The result of phytochemical analysis (in Table 2) applied to red cabbage and *Hibiscus sabdariffa* extracts showed that the extracts had different chemical structures. This analysis showed that the nucleus was stained by the flavonoid groups in red cabbage extract and the cytoplasm was stained by the phenolic structures in the *Hibiscus sabdariffa* extract.

In TLC analysis, different color discrimination was observed in *Hibiscus sabdariffa* and red cabbage extracts running in the same solvent. There are two plants and two different dye molecules as shown in Fig. 3. in the middle of the picture, the purple-blue dye of red cabbage was been leading. while the pink dye of *Hibiscus sabdariffa* was left behind in the first left.

Discussion

There are many tissue staining studies done with the extract of *Hibiscus sabdariffa* plant. In the study conducted by Basseyy et al, *Hibiscus sabdariffa* extract was used to investigate the sperm morphology of Sprague Dawley rats. Wistar rat testis and ovarian tissues were stained by an ethanol extract of *Hibiscus sabdariffa*. In this process which was applied directly to tissues at room temperature, and in 1 hour the cytoplasm had light purple staining [12]. In polar solvent and untreated tissue without chemicals before dyeing and without mordant, the dye was not permanent and the dyeing process was long. In this study, permanent light red ++ (medium) staining occurred in the cytoplasm in a shorter time by using a metal

salt in chloroform–water *Hibiscus sabdariffa* extract on tissues exposed to chemicals using routine H&E staining process steps (Dyeing process 2).

In another study, light and pale red staining occurred in the liver, kidney, heart, and lung tissues of Wistar albino rats with an aqueous extract without mordant of *Hibiscus sabdariffa* in the cytoplasm.³ Thus, in this study, it was decided to apply the *Hibiscus sabdariffa* on the same tissues as a cytoplasm dye. Water, alcohol and chloroform extracts of basil, alkanet and red cabbage were used to stain the nucleus in hematoxylin color. Then, iron sulphate, tin chloride and alum salts were added to the extracts, it was seen that the aqueous extract of red cabbage including the salt pair ($\text{FeSO}_4 \cdot 5\text{H}_2\text{O} + \text{alum}$) stained the nuclei of the liver and kidney tissues. The details of the other plant extracts were not included. Because there was no strong dual staining until the red cabbage. The red cabbage solution containing (ferrous and alum) salt pair after waiting for 1 week in a dark place, the nucleus appeared, and purple color intensity of the nucleus was increased. After testing *Hibiscus sabdariffa* and red cabbage extracts in individual tissues, routine H&E staining procedure steps were used to provide a dye combination similar to hematoxylin-eosin. The presence of different flavonoid dye groups in the dye solutions subjected to the same process has affected the dyeing results like shown in Fig. 3. Different terpenoid content of extracts was affected by the staining result. So, different regions on tissues were separately stained by two extracts. Thus, since the dye in the two extracts showed bounding with different places in the cell, *Hibiscus sabdariffa* extract stained cytoplasm. Red cabbage extract also stained the nucleus. Just like the difference in Hematoxylin and Eosin dye structures, they dyed different regions in the same tissue. In this study, a new natural dye pair (RC-HS) has been proposed instead of the Hematoxylin-eosin dye pair commonly used in medical laboratories. We hope that this will spread the use of natural dyes in medical fields outside of industrial areas.

Declarations

Funding

No.

Acknowledgments

I thank the Histology department of Erciyes University.

Author Contributions

NK: The author discussed the results and contributed to the final manuscript. The author carried out the experiments with helping of her students on September 2019.

Consent to Participate (Ethics)

This research is not involved human organs/tissues Research is only involved Wistar rat tissues. This study was approved by the Erciyes University ethics committee dated 16 November 2016 and numbered

16/144. Informed consent was obtained from all individual participants included in the study.

Consent to Publish (Ethics): Author is responsible for the correctness of the statements provided in the manuscript. This is not case study.

Declaration of interest

There is no conflict of interest.

Compliance with ethical standards, All the experimental protocol was approved by the Erciyes University Experimental and Clinical Research Center (DEKAM) ethics committee.

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Figures

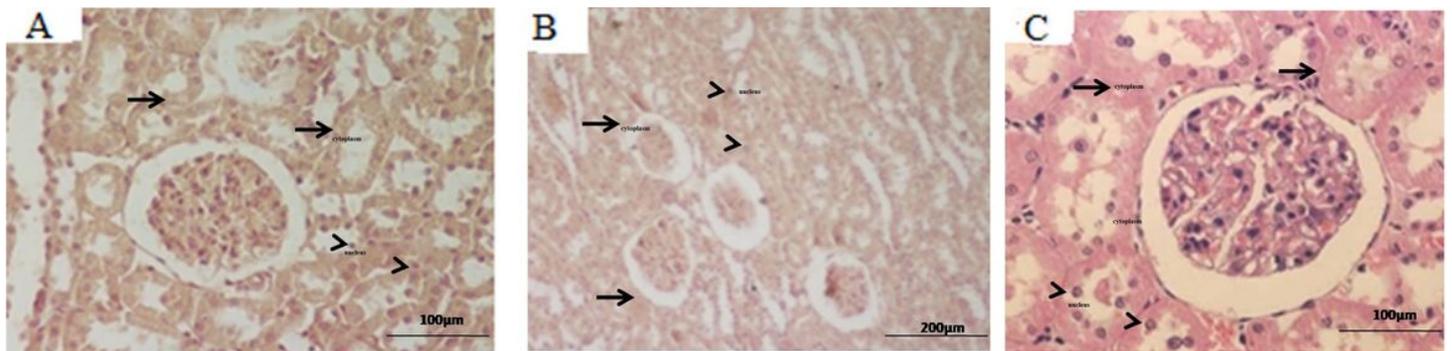


Figure 1

Histological morphology of rat tissue: A. Kidney tissue sample which underwent *Hibiscus sabdariffa* and red cabbage extract application, 200×; B. Kidney tissue sample which underwent *Hibiscus sabdariffa* and red cabbage extract application, 100×; C. Positive control with H&E, 100×

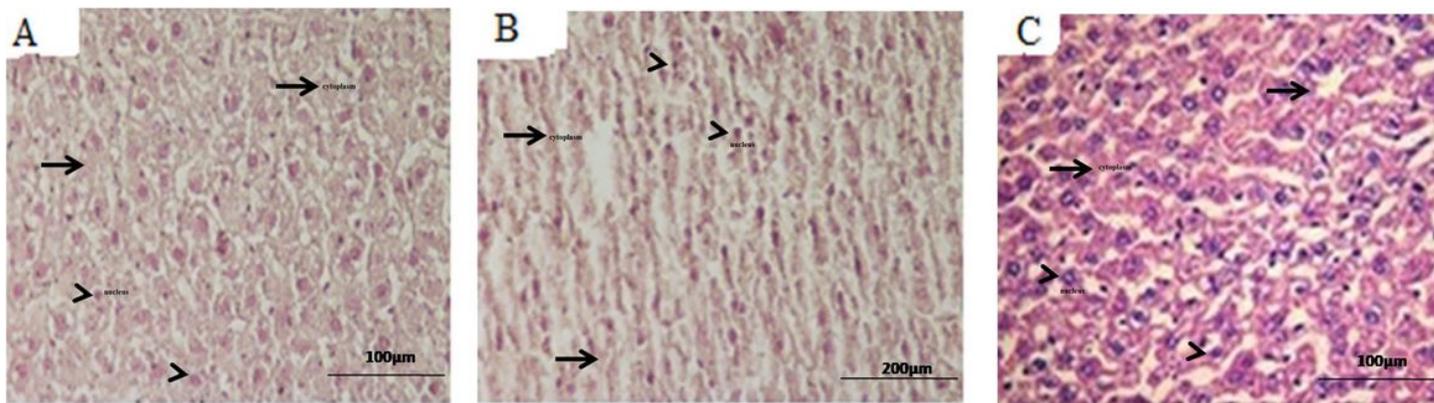


Figure 2

Histological morphology of rat tissues: A. Liver tissue sample which underwent Hibiscus sabdariffa and red cabbage extract application, 200x; B. Liver tissue sample which underwent Hibiscus sabdariffa and red cabbage extract application, 100x; C. Positive control with H&E. 100x

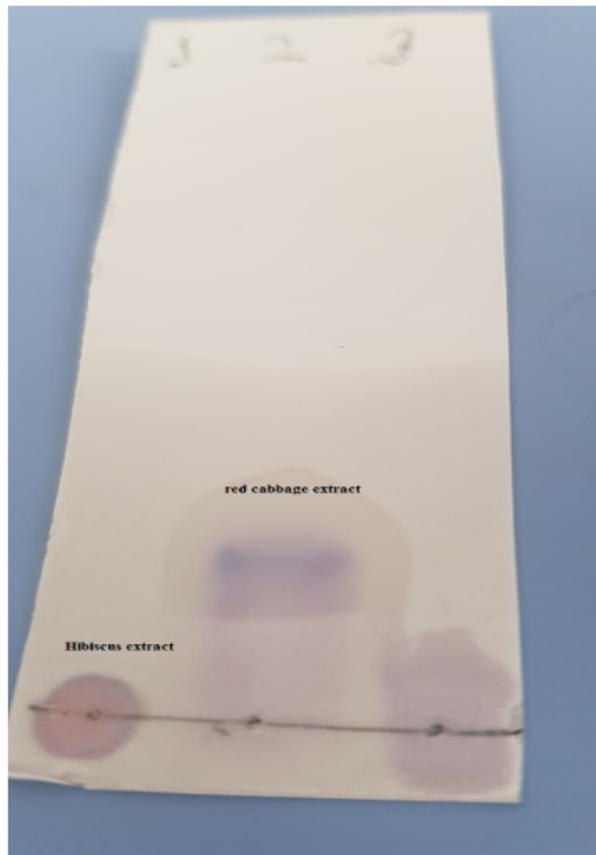
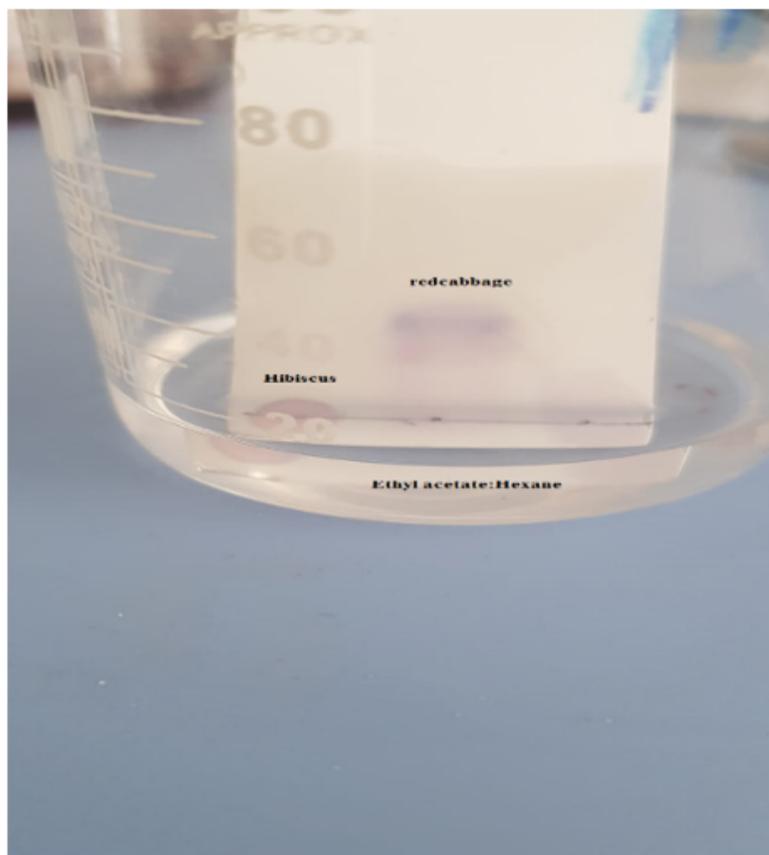


Figure 3

Photograph of TLC optimal solvent (OS) of Hibiscus sabdariffa and red cabbage extracts