Extraction and Characterization of A Chromosomal Stain From Black Mulberry (Morus Nigra)

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Abstract

A dark-blue chromosomal stain from Black Mulberry (Morus nigra) was extracted using organic solvents including dioxane, n-hexane, petroleum ether and acetone. This extracted stain stained chromosomes satisfactory. In order to ascertain purity of the extracted stain, it was compared spectrophotometery with three commercial standards stains; they are Azur II Eosin, Giemsa and Methylene blue Stains using a 200-900nm Spectrophotometric scan at three different concentrations (25, 50 and 100mg/ml).

It was found that the second peaks produced by Azur II eosin and Giemsa stain (as well as the single peak produces by Methylene blue) occupied the same area occupied by the second peak of the extracted stain, which agree to some degree of similarity. The extracted method proved to be an appropriate analytical technique with hope that further trials can be carrying out to simplify the extracting of the stain in future researches and make it less expensive.

Keywords: chromosomal stain, extraction, Mullberry

استخلاص وتوصيف صبغة كروموسومية من التوت الأسود

الخلاصة

تم استخلاص صبغة كروموسومية زرقاء قاتمة من التوت الأسود من خلال استخدام مذيبات عضوية مثل الديوكسان والهكسان وأيثر البترول والاسيتون، واستطاعت هذه الصبغة المستخلصة صبغ الكروموسومات بطريقة مُرضية، وتم تقييم الصبغة طيفياً بالمقارنة مع ثلاثة صبغات تجارية قياسية أخرى هي أزور –II أيوسين، وصبغة كيمزا، وصبغة مثيلين بلو في عدة تراكيز (25، 50، 100 ملغم/مل) بإستخدام جهاز المطياف ما بين 200-900 نانومتر، ولقد وجد أن القمة الثانية الناتجة من أزور –II أيوسين، وصبغة كيمزا (فضلاً عن القمة الوحيدة في صبغة مثيلين بلو) تحتل المساحة نفسها التي تحتلها القمة الثانية من الصبغة المستخلصة ، مما يجعل نوعاً من التشابه بين الصبغات، ويمكن عد طريقة الاستخلاص طريقة تقنية تحليلية ، مع الأمل في امكانية اختصار هذه الطريقة وتبسيطها في المستقبل وجعلها أقل كلفة في التجارب القادمة.

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Introduction

Due to the lack and high prices of several important cytoplasmic and chromosomal stains from local markets in 1990s, attempts were made to produce such stains from local sources. The extraction of a chromosomal stain from local sources such as insects and plants proved to be a very difficult task. More than sixty attempts were tried since 1995 to produce a chromosomal stain, but most extractions that were done proved unsatisfactory. While they were able to stain the skin, hair and textiles and even can be used as watercolors, but none have the ability to penetrate the supercoiled structure of the DNA and the thick layer of water surrounded it (1, 2). At last and out of many unsuccessful trials, extracts from Black Mulberry *(Morus nigra)* show promises during primary trails and the aim of this research was to extract a dark-blue chromosomal stain from Black Mulberry.

Materials And Methods

Black Mulberry (*Morus nigra)* was identified by Department of Horticulture, College of Agriculture, University of Baghdad. The fruits collected from trees in the researchers' gardens or bought from market were used as a local source for preparation of the extract. The fruits were dried, weighed and powdered before use.

Extraction of the stain

The classical method of Smith and Daniels (3) was used. According to this method, the harvested fruits were dried quickly at moderate temperatures then powdered by mortar and piston. The 100 gm of powdered fruits were defatted by extraction with 200 ml 1,4-dioxane in a soxhlet apparatus and then were dried at room temperature. The dried powder was mixed with 300 ml *n*-hexane and left for one week in the dark for complete precipitation of the stain. After that, the solution was centrifuged at 4000 x g for 30 min. The pellet was washed with petroleum ether (b.p. 40-60ºC) several times, centrifuged as above, then washed twice with acetone to remove all residues of petroleum ether, then centrifuged as above. Methanol added twice to remove traces of acetone and centrifuged each time. The final dark-bluish powdered pellet (2.5 gm) was collected under suction through filter paper Whatman No.1, kept in the dark at 4ºC and used as a crude stain at later experiments.

Preparing a karyotype

A karyotype was prepared according to Chattopadhyay *et al.* (4) as modified by Hliscs *et al.* (5). A sample of peripheral blood (5 ml) is drawn and coagulation was prevented by the addition of sodium heparin. Mononuclear cells (lymphocytes and monocytes) were purified from the blood by centrifugation at low speed (4000 x g) which allow mononuclear cells only to remain in the supernatant.

The mononuclear cells were cultured for 72 hr in the presence of mitogen such as phytohemaggultinin (PHA) which stimulates the lymphocytes to proliferate (6, 7). At the end of the incubation period, the culture was treated with colchicine for 40 min which disrupts mitotic spindles and prevents completion of mitosis (8). This greatly enriches the population of metaphase cells. The cells then

resuspended in 0.075M KCl hypotonic buffer for 15 min at room temperature. This makes the nuclei swell osmotically and greatly aids in getting preparations in which chromosomes do not lie on top of each other (9, 10, 11).

A small volume 3:1 methanol: acetic acid fixative was added to the cell suspension and three more fixative washes were subsequently performed. Cell pellet(s) were stored at -20ºC in a fixative.

Slide preparation

Each glass slide scraped and washed carefully to remove the wax layer from it, then all slides autoclaved for 20 min at 121°C, and placed at room temperature prior to use. Using an automatic pipette, according to the method of Henegariu *et al.* (12), 25-35µl cell suspension was distributed by moving the pipette tip parallel to the surface of the slide gently (instead of dropping it from 2cm high) and excess liquid was drained on a paper towel. When the surface of the slide became grainy (as the fixative evaporated), the slide was placed face-down in the steam of a hot water bath (70-80ºC) for 1-3 sec, then was dried by placing it on a metal plate since hot steam overpowered any potential influence of the atmospheric humidity. Higher temperatures were used (≈90ºC) to increase chromosome spreading (personal observations). After overnight incubation at 65 ºC (aging), G-banding was performed.

G-banding

G-banding was performed according to the classical method described by Moorehead *et al.* (10) as modifies by Hirsch *et al.* (11). Slides were incubated for 3 sec to 1 min in trypsin solution (0.1g trypsin in 100 ml isotonic buffer). Slides were rinsed in isotonic buffer and incubated for 1.5-5 min in a Coplin jar with Giemsa stain. Giemsa solution (or extracted stain from *M. nigra*). After staining, slides were rinsed few seconds each in phosphate buffer and water and then air-dried. Slides were examined under microscope with a 10x or 20x objective and photos were taken using a digital camera.

Staining with the extracted stain

0.1 gm of the extracted stain in 10 ml methanol (10mg/ml) was used to stain chromosomes with the addition of trypsin.

Spectrophotometer was used for scanning different dyes between 200-900nm.

Giemsa stain was prepared by mixing 1.5 ml Giemsa with 50 ml phosphate buffer. Azur II Eosin was prepared from 5g solid stain and 100ml of distilled water. Methylene blue was prepared from 0.5g in 100ml distilled water, 30ml ethanol and 1ml of 10% KOH (12).

Results And Discussion

The extracted stain from *M. nigra* consists of dark-blue crystals with about 10% white crystals mixed with it. The stain's crystals were stable for 3-4 months at room temperature in the dark, and must protected from moisture because the staining reaction is oxidative, therefore, the oxygen in the solution will initiate the reaction and ruin the stock stain. The aqueous extracted stain is good only for 1 day.

Cells from peripheral blood are used since they have several advantages: they are easy to collect, can be cultured rapidly (three days or less)

and only a few drops of blood are necessary for analysis (13, 14, 15).

The protocol used by the researchers allows better chromosome spreading through the use of precise timing and learning from the experiences of previous researchers (7, 10, 13, 16, 17). The extracted stain stained chromosomes at 10mg/ml concentration with the addition of trypsin, with Giemsa stain as a control. No visible bands were seen clearly when trypsin was added. Chromosomes stained with Giemsa stain and Eosin Azur II was used for comparison (Fig. 1).

The extracted stain was compared spectrophotometery with three stains; Azur II Eosin, Giemsa's and Methylene blue Stains at 200-900nm Spectrophotometric scan at two different concentrations (25 and 50mg/ml) to ascertain its purity.

The intensity of peaks can be affected by dilution, pH, concentration strength and temperature (18, 19, 20, 21).

Both Giemsa and *M. nigra* extracted stains showed the same peaks at 540nm and 670-690nm at 25 and 50mg/ml concentrations. In the same time, Azur II Eosin has two peaks at 520nm and at 630-640nm, while Methylene blue stain has a single peak at 610-640nm at 25 and 50mg/ml concentrations (Figs. 2, 3, 4).

By comparing the peaks of the four stains, it was found that only Giemsa stain has occupied the same areas occupied by *M. nigra* extracted stain, while the peaks of the other two stains show different characteristics. Therefore, the authors assume that Giemsa stain shares many characteristics with the extracted stain, but whether they are the same or not, this needs a further work.

The aim of this research of obtaining a chromosomal stain from local source was achieved successfully. The cost of the extraction can be reduced greatly by using commercial solvents instead of highly purified solvents.

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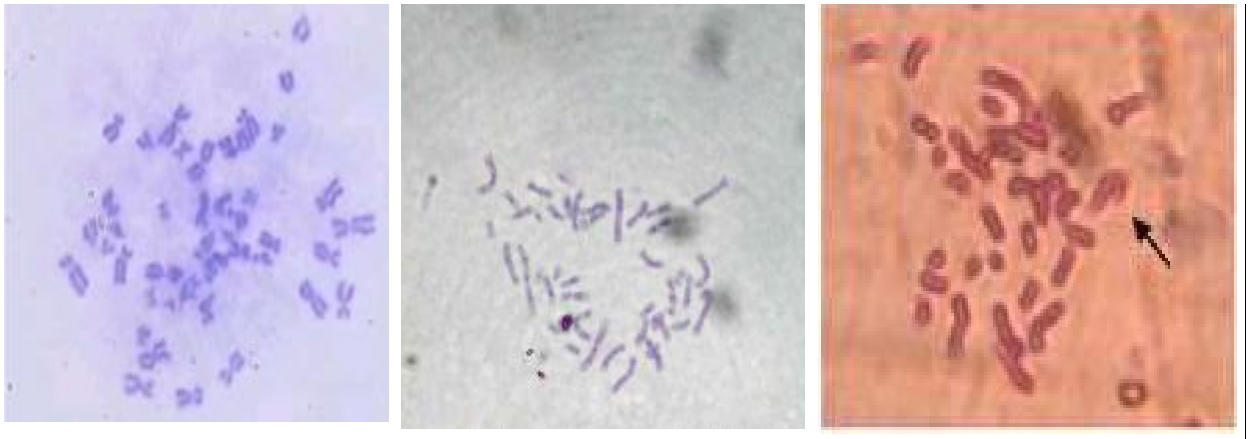


A **B** **C**

Figure) Human blood chromosomes stained by *a) Morus nigra* extracted stain, b) Giemsa stain, c) eosin-Azur II stain

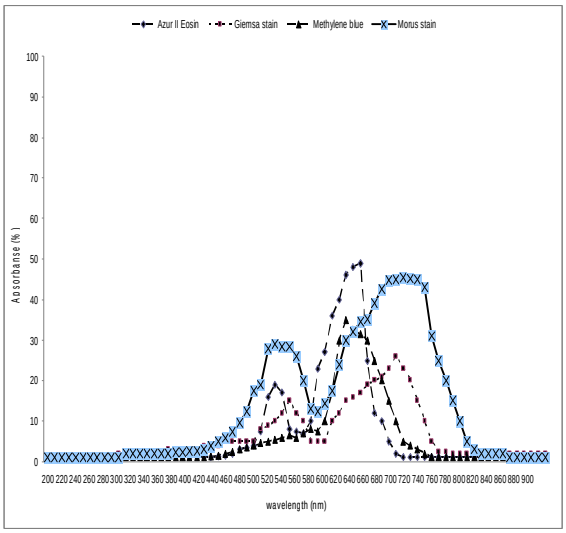
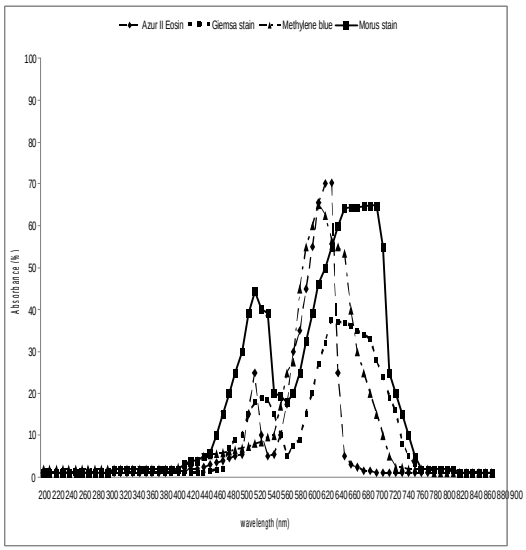
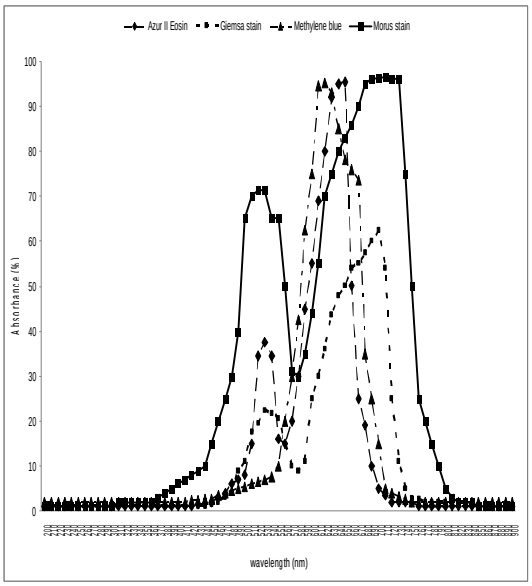


Figure (2) A 200-900nm spectra comparison between Azur II Eosin stain, giemsa stain, Methylene blue stain, and *Morus nigra* extracted stain at 25 mg.ml^{-1} concentration



Figure(3)A200-900nmspectra comparison between Azur II Eosin stain, giemsa stain, Methylene blue stain, and *Morus nigra* extracted stain at 50 mg.ml^{-1} concentration



Figure(4)200-900nm spectra comparison between Azur II Eosin stain, giemsa stain, Methylene blue stain, and *Morus nigra* extracted stain at 100 mg.ml^{-1} concentration