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Extraction of Aloin from *Aloe Vera* plant and study its effect in Micronucleus Formation in Acute Lymphoid Leukemia

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Abstract:

Aloin extracted into alcohol-rich phase with high extraction efficiency, meanwhile majority polysaccharides, proteins, mineral substances and other impurities were extracted into salt-rich phase. Partitioning of AQs[Anthraquinones] is dependent on hydrophobic interaction, hydrogen bond interaction, and salting-out effect in Aqueous tow –phase system [ATPS]. Aloin was partially purified by using 1-propanol [NH_4] $2SO_4$, the use of this solvent showed high efficiency 90.61% compared with other solvent [2-propanol and ethanol]. The concentration of aloin detected by HPLC technique, which reached to 91.84% as focus turns out that there is compatibility between the sample and the standard in shape and retention time which reached [1.462] compared with the standard [1.465]. After the treatment of blood leukemic patient cultures, three concentrations of aloin were used [100, 200 and 400] µg/ml, the last concentration showed the ability to reduce the coefficient of micronucleus formation to [0.0062] micronucleus/cell, compared with non- treated culture which reached to [0.0236] micronucleus/cell.

استخلاص الألوين من نبات الصبار ودراسة تأثيره في معامل التكون النوى الصغيرة لمرضى سرطان الدم الحاد.

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الخلاصة

استخلص الألوين بكفاءة عالية بالطبقة الكحولية بينما السكريات والبروتينات والمواد المعدنية وغيرها من الشوائب تم استخلاصها في الطبقة الملحية. ان عملية فصل الأنثراكوينون تعتمد على تفاعلات الهيدروفوبك وتفاعلات الأواصر الهيدروجينية وتأثير التمليح الخارجي.

نقي الالوين جزئيا باستخدام نظام 1–propanol [NH_4] $2SO_4$ ، اذ أظهر امكانيه عالية مقارنة في المذيبات الأخرى [الأيثانول،البروبانول الثنائي] اذ بلغت الكفاءة ٩٠.٦١% .

قدر تركيز الألوين بوساطة تقنية HPLC اذ بلغ التركيز ٩١.٨٤% كما تبين ان هنالك توافق عالي بين عينة الأختبار والمركب القياسي للألوين في الشكل ووقت الأحتجاز والتي بلغت للعينة ١.٤٦٢ بالمقارنة مع المركب القياسي ١.٤٦٥.

عند معاملة مزارع الدم للمصابين بمرض ابيضاض الدم بثلاثة تراكيز من الالوين [[400 and 200 ,100 µg/ml حيث اظهر التركيز 400 µg/ml القدرة على خفض معامل تكون النوى الصغيرة الى ٠.٠٠٦٢ micronucleus/cell مقارنة مع المزارع الغير المعاملة 0.0236 micronucleus/cell .

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Introduction:

Aloe vera is one of the medicinal plants have been used as ethnic medicines in many different countries for centuries, possessing functions, such as anti-cancer, anti-inflammatory, anti-virus, evacuating, protecting liver, and increasing immunity[1].

Aloe vera L. has been most commonly used as medicine, healthy foods, and cosmetics nowadays. The active components of aloe include anthraquinones, chromones, polysaccharides, enzymes and, so on [2]. It is reported that anthraquinones and chromones are responsible for anti-cancer activity, anti-inflammatory, and evacuating [3]. Aqueous two-phase system was first introduced by Albertsson [4]. It has gained increase attention for purification and separation. purposes particularly in the biotechnology field because of its biocompatibility and high capacity [5]. In recent years, as a novel liquid–liquid extraction technique, alcohol/salt ATPS has many advantages such as low cost, low viscosity, easy recovery of alcohol by evaporation and easy to scale-up in industrial production [6,7]. In this paper, we developed a simple, efficient and cheap technique for the purification of Aloin from Aloe vera, Aloin are one kind of major active ingredients in aloe leaves, which have multiple pharmacological activities including laxative, anti-bacterial, anti-oxidant, hemostatic and anti-cancer [8,9].

Aims of study:

The present project was designed to evaluate the immunoIogicaI and cytogentic effects of Aloin extracted from *Aloe vera* plant in albino male mice and the micronucleus formation in leukemic patients.

Materials and methods:

Plant Characterization and Identification:

Aloe vera was collected from Baghdad University gardens and local market [University of Baghdad, college of science, Biology department] identified the plant as *Aloe vera*.

Plant Collection and crud Anthraquinones extraction:

Plants leaves 23.419kg of aloe vera were collecting for the period November to March, these leaves were cut near the thick end and washed with Fresh tap Water, then scratched and placed in glass plates at a straight position for the descanting of crud aloin for 24 hours.

Partial purification of crud Anthraquinones:

Drying the Extract:

Crud Anthraquinones placed in petri dish and incubated at 40°C. for 24 hours and then Skimming the Aloe crystals powder.

Preparation of crud Anthraquinones [AQS] to salting out:

Aloe peel powder was soaked in 60 % ethanol solution for 24 h. The suspension was separated by a centrifuge at 4,000 rpm for 10min. The colloidal part was separated from the clear solution, then Sulfuric acid solution and chloroform were added to the extract and then refluxed. The chloroform extract was removed by a separating funnel; a yellowish-brown colloid was the crude extract obtained after evaporating the chloroform, and then dissolved in methanol for further studies [10].

Preparation of alcohol/salt Aqueous Two Phase System [ATPS]:

Five mL distilled water, 2.0 mL alcohol, a given amount of salt and 0.1 mL aloe AQs solution were added to. The mixture was stirred well to make the salt dissolve completely. The separation phase could be achieved during a few seconds, two clear phases formed. The uper phase was mainly composed of alcohol and AQs with a small volume, and the lower phase was the salt-rich solution containing impurities with large volume. The volume of each phase was noted down. The alcohol-rich phase was withdrawn to another tube using a syringe, then diluted using 0.5 % magnesium acetate–methanol solution, the AQs solution turns to red color under this weakly basic condition. The total AQs concentration was determined by colorimetric method using a UV–Vis spectrophotometer.

The AQs concentration in the salt-rich phase was determined by mass balance[10].

The phase ratio [R] is defined in Eq.:

- $R= V_t / V_b$

  where Vt and Vb are the volumes of alcohol-rich phase and salt-rich phase, respectively. The partition coefficient [Kd].

  is defined in Eq. :

- $K_d= C_t / C_b$

where Ct and Cb are the AQs concentrations in the alcoholrich phase and salt-rich phase, respectively. The extraction efficiency [E] of AQs in the alcohol-rich phase is determined from Eq.:

- $E= K_d / [K_d +1/R]*100\%$

Phase diagram:

The diagram phase was prepared by a turbidimetric titration method [11]. The phase diagram could provide information about the concentration of phase-forming components required to form an ATPS. Firstly, alcohol of known mass was added into a 10 mL graduated cylinder. A solution of known mass fraction of a salt was added drop wise and then the mixture well mixed. The solution was clear at first, but after a certain amount of the salt solution added, one further drop made the mixture turbid, which then separated into two phases. The composition of each component was noted down. Then, a few drops of water were added to make the mixture clear again, and the above procedure was repeated to obtain sufficient data to construct the diagram phase.

High performance liquid chromatography [HPLC]:

The procedure was done according [12]. The partial purified and standard sample was measured by HPLC at 254nm. The mobile phase was 0.01µ ammonium phosphate buffer. The concentration of the active material [aloin] was calculated according to the following equation:

$$\text{The concentration } \% = \frac{AUC\ [test]}{AUC\ [standard]} * 100$$

AUC= Area Under Curve.

Aloe Doses and Concentrations:

In human cultures, the extract was assessed, in which, three concentrations [100, 200 and 400 µg/ml] of the aloin extract were tested [13].

Micronucleus Formation in the Blood of Leukemia Patients:

Subjects:

The subjects of this assay were patients [5 subjects] with acute lymphoid leukemia [ALL], which were referred to the [AL Yarmook Hospital]. The diagnosis was based on a clinical examination and laboratory evaluations, which were carried out by the consultant medical staff at the hospital. The patients were Iraqi, and their age range was 25-40 years and none of them was under treatment. A further five healthy subjects [control group] were also investigated.

They were university staff and students that had no history or signs of leukemia, and matched with patients for ethnic background and age.

Peripheral blood [5 ml] was obtained under aseptic conditions from each subject by a venepuncture using a disposable syringe pre coated with heparin. The blood sample was placed in a cool box and transferred to the laboratory.

Treated Groups:

This experiment was designed to assess *in vitro* the effects of the concentration of Aloin extract [100, 200 and 400 µg/ml] in the micronucleus formation in the cultured blood cells of patients and controls. Therefore, three cultures were set–up for each subject:

- **Culture Number 1**: Blood culture of [Healthy and Leukemia patients] each of which was treated with 100 µg/ml of Aloin extract.
- **Culture Number 2**: Blood culture of [Healthy and Leukemia patients] each of which was treated with 200 µg/ml of Aloin extract.
- **Culture Number 3:** Blood culture of [Healthy and Leukemia patients] each of which was treated with 400 µg/ml of Aloin extract.
- **Culture Number 4**: untreated culture [negative control].

Micronucleus Test Cultures:

The procedure of [14]was followed, in which, 2 ml of RPMI-1640 culture medium and then 0.5 ml of blood was added to the culture with 0.4 ml of Aloin, The Tree cultures were incubated at 37°C for 72 hours.

After 72 hours incubation, the culture tubes were centrifuged [800 rpm] for 5 minutes, and then the supernatant was discarded and the cell deposit was gently suspended in 5 ml of a warm [37°C] hypotonic KCl solution [0.1M]. The cell suspension was incubated in a water bath [37°C] for 30 minutes with a gentle mixing every 5 minutes. Then, the suspension was centrifuged [800 rpm] for 5

minutes, and the supernatant was discarded. The deposit was suspended in a few drops of a cold fixative [4°C], and the volume was made up to 5 ml with the fixative. The fixed cell suspension was incubated in the refrigerator [4°C] for 30 minutes, and after that, it was centrifuged [800 rpm] for 5 minutes. The process of fixation was repeated two times, and by then the cells was suspended in 1 ml of the fixative. The fixed cells were smeared on a clean slide, and left for air-drying. The slide was stained with Giemsa stain for 15 minutes, and then rinsed with distilled water, and finally it was air-dried.

The slide was examined under oil immersion lens [100X], and the cells were inspected for the formation of micronucleus. A total of 1000 cells were randomly examined, and the micronucleus index was scored using the following equation:

$$\text{Micronucleus index [micronucleus/cell]} = \left(\frac{\text{Number of Micronuclei}}{\text{Total Count of Cells}} \right) \text{x } 100$$

Results and discussion:

Partial Purification of Aloin from plant crud extracts:

The extraction efficiencies of different alcohol / salt ATPSs are shown in Table-1, it can be seen that 1-propanol/$[NH_4]2SO_4$ system has the highest extraction efficiency than other alcohol/salt systems. To optimize the $[NH_4]2SO_4$ concentration, 1.5–3.0 g [18.50–31.23 %, w/w] $[NH_4]2SO_4$ was added into the system. The alcohol/salt system with 17.84 % 1-propanol and 26.66 % $[NH_4]2SO_4$ has the best extraction ability, the extraction efficiency increased with $[NH_4]2SO_4$ concentration, it can be explained that the increase of $[NH_4]2SO_4$ will strengthen the salting-out effect and lead to increase the 1-propanol concentration in the alcohol-rich phase. The phase separation of ATPS is widely regarded as the result of the salting-out effect of salts in the alcohol/salt system [15]. When the salt concentration reaches saturation, the continuous addition of salt will be precipitated out and the extraction efficiency will not be increased. Moreover, due to the hydrophobicity of AQs, the hydrophobic interactions between AQs and alcohol-rich phase probably are the main driving forces for the extraction [16].

Table 1-Extraction of aloe AQs in different alcohol/salt ATPSs [the system contains 2.0 mL alcohol, 5.0 mL water, a given amount of salt and 0.1 mL crude AQs extract solution].

Alcohol/salt system	Concentration of alcohol/salt [%, w/w]	Phase ratio [R]	Partition coefficient [Kd]	Extraction efficiency [E, %]
1-Propanol / $[NH_4]2SO_4$	19.82/18.50	0.383	8.83	77.19
	19.12/21.41	0.393	9.25	78.45
	18.46/24.12	0.344	11.85	80.29
	17.84/26.66	0.338	64.35	90.61
	17.27/29.01	0.324	66.84	90.58
	16.73/31.23	0.300	65.25	90.14
2-Propanol/$[NH_4]2SO_4$	19.45/18.59	0.389	8.77	77.38
	18.76/21.51	0.393	21.04	84.22
	18.11/24.22	0.344	18.05	81.12
	17.50/26.76	0.323	22.57	80.94
	16.94/29.13	0.322	14.30	82.23
Ethanol/$[NH_4]24$	18.84/21.48	0.448	1.68 ± 0.02	42.97±0.47
	18.40/23.31	0.441	2.51 ± 0.13	52.95±0.19
	17.98/25.06	0.433	3.77 ± 0.08	62.06 ± 0.12
	17.58/26.73	0.419	5.39 ± 0.16	69.34±0.22

Estimation the Aloin purity by using the High Performance:
Liquid Chromatography [HPLC] technique:

The work was carried out at the Ministry of Science and Technology Department of Materials Research, Dr. Fadel Mohsen. For certification of the previous characterization and for determination

the degree of Aloin sample partial purity and comparison with standard one this analysis was done. The result of this test showed there was close compatible curves for the sample with that of standard one in the shape, but slightly different in the retention time, The partial purified Aloin sample appeared at [1.462] minutes while standard Aloin at [1.465] minutes as shown in Figure-1, from these results, the concentration ratio of Aloin tested sample was calculated, which was reached 91.84%, and these results indicated the high purity of the tested sample. And these results agreed with [12].

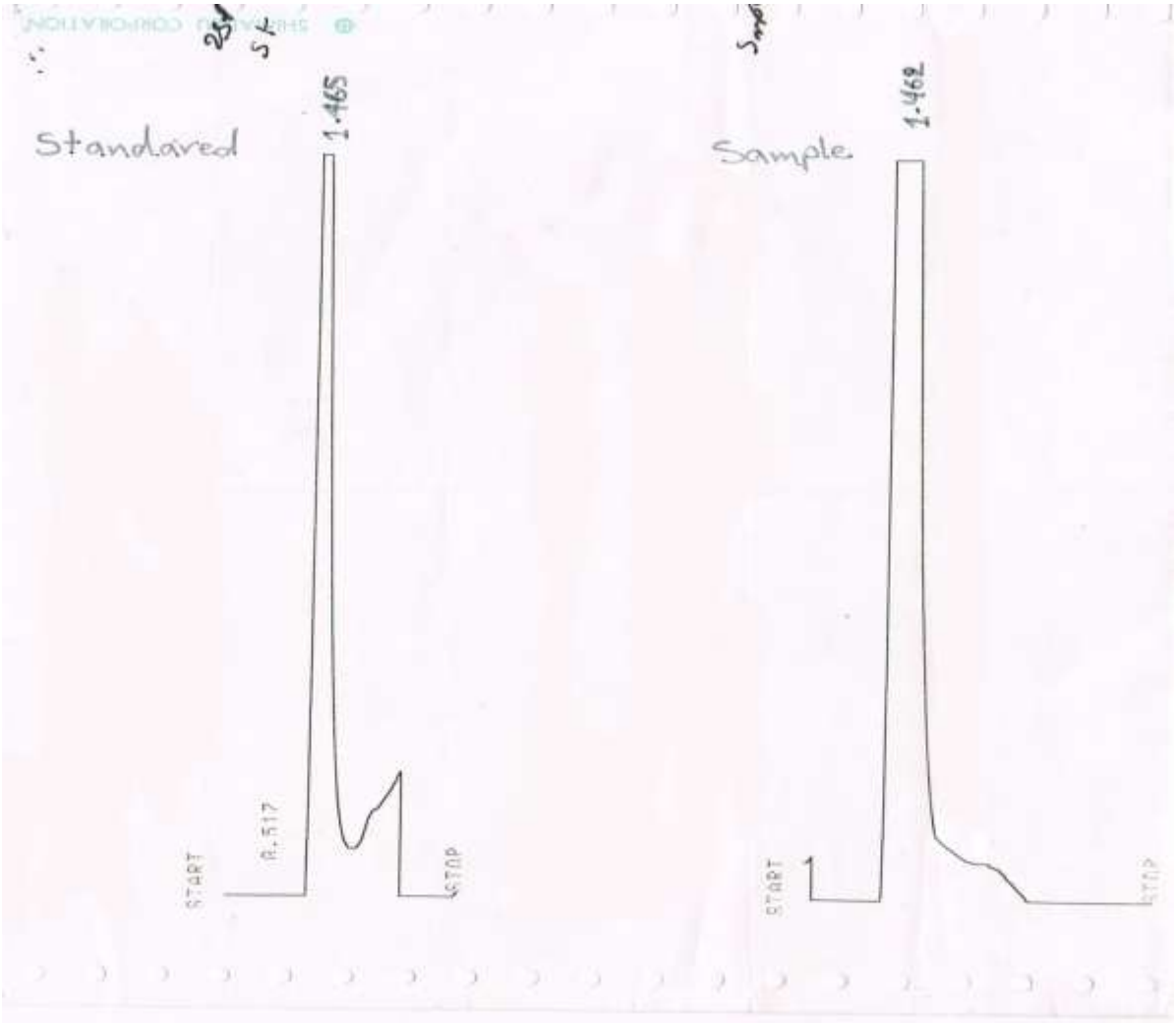

Figure1- the Aloin Standard and Sample Curves using the High Performance Liquid Chromatography [HPLC] technique at absorbance 254 nm.

Micronucleus Formation in Leukemia Patients:

The micronucleus formation Figure-2 was assessed in acute lymphoid leukemia [ALL] patients through a series of blood cultures that were detailed in the materials and methods and the results are given in Table-2:

- **Untreated Cultures**: The leukemia patients showed a significantly decreased frequency of micronucleus formation as compared to healthy controls.
- **Cultures Treated with Three Concentrations of Aloin Extract:** In healthy controls, the three concentrations [100, 200 and 400 µg/ml] of Aloin were significantly effective in reducing the spontaneous formation of micronuclei as compared to the corresponding untreated cultures. Blood cultures of leukemia patients treated with the same concentrations of the extract also showed

  a significant reduced frequency of micronucleus formation as compared to the corresponding untreated cultures.

Table 2- Micronucleus formation in lymphocyte cultures [treated with Aloin extract] of acute lymphoid leukemia patients and healthy controls

Groups	Concentration [µg/ml]	Micronucleus/cell [Mean ± Standard Error]		Probability ≤
		Healthy Controls	Leukemia patients	
control [untreated]	0.0	0.0140±0.0011	0.0236±0.0023	0.05
Aloin extract	100	0.0104±0.0002	0.0144±0.0009 38.98	0.05
	200	0.0076±0.0002	0.0098±0.0009 58.47	0.05
	400	0.0038±0.0005	0.0062±0.0004	0.05

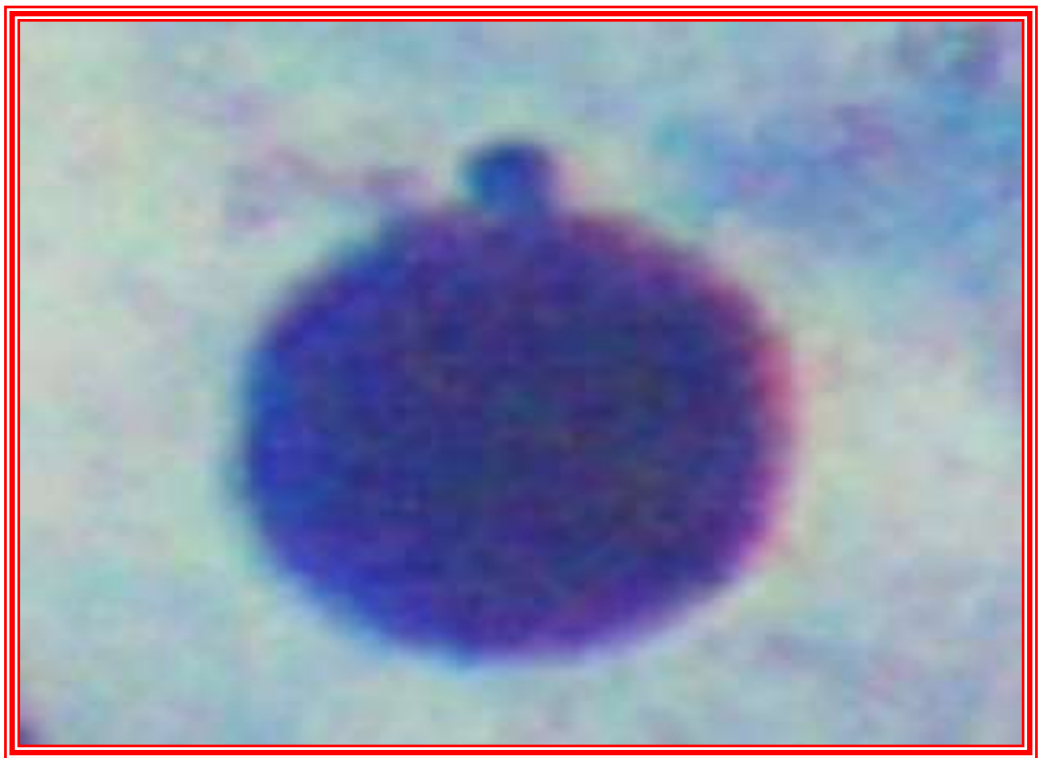

Figure 2- Micronucleus formation in acute lymphoid leukemia [100X].

The result of genetic evaluation showed that a treatment with Aloin extract was associated with a significant reduction in micromucleus formation, Such finding can be considered important, especially if we consider that most cancers are preceded by mutations induced by different agents, especially those that have oxidant effect, Numbers of studies have been conducted in regard to antioxidant activity of *Aloe vera* and in general their finding are in agreement with the present result [17].

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