Assessing the Anti-mutagenic Potentials of Sage *Salvia officinalis* L. Leaf Aqueous Extract in Cultured Blood Cells of Acute Lymphocytic Leukaemia Patients Using the Micronucleus Formation Assay

تقدير الفعل المضاد للتطفير للمستخلص المائي لأوراق نبات الميرمية في خلايا المزارع الدمية لمرضى ابيضاض الدم اللمفي الحاد باستخدام فحص تكون النوى الصغيرة

Ruqaya M. Al-Ezzy Khulood W. Al-Samarraei* Ali H. Ad'hiah**

College of Science\ Al-Nahrain University.

*Biotechnology Research Center\ Al-Nahrain University.

** College of Science\ Baghdad University

رقية محمد العزي خلود وهيب السامرائي * علي حسين أدحية**

كلية العلوم/ جامعة النهرين

*مركز بحوث التقنيات الإحيائية / جامعة النهرين

** كلية العلوم/ جامعة بغداد

Abstract

Three concentrations (250, 500, 1000) µg/ml of sage *Salvia officinalis* L. leaf aqueous extract were assessed for their anti-mutagenic potentials in cultured blood cells of 10 acute lymphocytic leukaemia (ALL) patients and a similar number of apparently healthy controls. The parameter of assessment was micronucleus (MN) formation, which was either spontaneous or induced by a treatment with the mutagen cytostar 125 µg/ml, and accordingly, eight cultures were set-up. Blood cells in culture I was negative control (untreated cells), while in culture II, the cells were treated with the mutagen cytostar (cytarabine) at a concentration 125 µg/ml. Cultures III, IV and V were treated with the three concentrations of sage extract respectively, while in cultures VI, VII, VIII, interactions between the extract (the three concentrations respectively) and cytosar were carried out. The results demonstrated that ALL patients showed a significant increased frequency of MN formation in the eight cultures as compared to the corresponding cultures in healthy controls. With respect to the spontaneous formation of MN formation (cultures III, IV, V), the three concentrations of sage extract demonstrated a gradual significant reduction of MN frequency in patients (0.0144, 0.0098, 0.0062) MN/cell, respectively and control subjects (0.0104, 0.0076, 0.0038) MN/cell, respectively. In the induction cultures (VI, VII, VIII), a similar reduction was observed and the dose 1000 µg/ml exerted the highest reduction in both patients and controls (0.0108, 0.0086) MN/cell, respectively. These results demonstrate the anti-mutagenic importance of sage leaf aqueous extract.

المستخلص

قدَر الفعل المضاد للتطفير لثلاث تراكيز (250 ، 500 ، 1000) مايكروغرام/مل من المائي لأوراق نبات الميرمية في خلايا المزارع الدمية لعشرة من مرضى ابيضاض الدم اللمفي الحاد وعدد مماثل من أفراد السيطرة الأصحاء ظاهريا ، وكان تكون النوى الصغيرة هو العامل في هذا التقدير والتي كانت تلقائية أو مستحثة عند المعاملة بالمطفر سايتوسار (125 مايكروغرام/مل) . وفي ضوء ذلك أعدت ثمانية مزارع خلوية للدم . كانت خلايا المزرعة I سيطرة سالبة (غير معاملة) ، في حين شملت المزرعة II خلايا معاملة بالمطفر سايتوسار بتركيز 125 مايكروغرام/مل . وتمت معاملة المزارع III، IV، V بثلاث تراكيز من مستخلص الميرمية على التوالي ، في حين جرى تداخل مابين هذه التراكيز وسايتوسار في المزارع VI، VII، VIII

على التوالي . أفصحت النتائج عن زيادة معنوية في تكرار النوى الصغيرة لمرضى ابيضاض الدم الحاد مقارنة بأفراد السيطرة وللمزارع الخلوية الثمانية . وفيما يخص التكون التلقائي للنوى الصغيرة المزارع (III،IV V)، فكان للتركيز الثلاث لمستخلص الميرمية قابلية تدريجية معنوية في خفض تكرار النوى الصغيرة في المرضى (0.0144 ،0.0098 ،0.0062) نواة صغيرة على التوالي وأفراد السيطرة (0.0104 ،0.0076 ،0.0038) نواة صغيرة على التوالي . أما في المزارع المستحثة المزارع(VIII، VII، VI)، فقد لوحظ نمطا مماثلا من الاختزال إلا إن الجرعة 1000 مايكروغرام/مل هي الأفضل سواء كان ذلك في المرضى أو السيطرة (0.0198 ، 0.0068) نواة صغيرة على التوالي . تشير هذه النتائج إلى الأهمية المضادة للتطفير لمستخلص أوراق نبات الميرمية المائي .

Introduction

Herbs are plants or plant parts that are valued for their medicinal and savory qualities. They contain and can produce a variety of chemical substances that have different biological actions, with a special reference to their medicinal importance, and according to the world health organization (WHO), about three-quarters of the world population relies upon traditional remedies (mainly herbs) for the health care of its people [1]. Sage *Salvia officinalis* L. is one of these medicinal plants, and researches have revealed its anti-mutagenic and anti-carcinogenic potentials, and such medicinal implications have been ascribed to more than 160 types of phenolic compounds that have been described in different species of the plant [2]. It is shrub-like plant, which is classified under the family Lamiaceae [3]. Sage is a further subject of medicinal plant researches, and in folkloric medicine, the plant is advised to be used in the treatment of colds, abdominal pain and fever, while in anti-mutagen and anti-carcinogen evaluations, the plant chemical compounds have shown promising results [4, 5].

Several *in vitro* and *in vivo* assays are employed for the evaluation of mutagenecity; for instance, structural chromosomal aberrations, and sister chromatid exchanges, sperm-head abnormalities and micronucleus (MN) formation, each with its importance in such evaluation [6]. The latter assay and since its introduction in 1959 by Evans and colleagues has attracted the investigators due its sensitivity in the assessment of mutagenic effects induced by different mutagens [7, 8]. Assessment of mutagenesis represents important evaluation of DNA damage by endogenous and environmental agents, especially if we consider that most malignant cells have undergone mutagenic changes before their carcinogenic transformation [9]. In this regard, acute lymphocytic leukaemia (ALL) has largely been considered as a malignancy in which environmental factors are suspected in its aetiology, and such factors are able to induce mutation(s) in genetically predisposed individuals, and finally consequence in a malignant transformation when the DNA-repair mechanisms fail in repairing these changes [10]. Accordingly, the present investigation was planned to assess the *in vitro* potential role of sage leaf aqueous extract in monitoring the frequency of MN formation in the blood cells of a sample of Iraqi ALL patients.

Subjects, Materials and Methods

i. Subjects

The subjects were 10 ALL patients, who were referred to the Baghdad Teaching Hospital for diagnosis and treatment. 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 Arabs, and their age range was (25-40) years. They were firstly diagnosed (January-April, 2006), and none of them was under treatment. A further 10 apparently healthy subjects (control group) were also investigated. They were university staff and students who had no history or signs of leukemia, and matched with patients for ethnic background and age. Peripheral blood 5ml was obtained under aseptic conditions from each subject by a venepuncture using a disposable syringe precoated with heparin. The blood sample was placed in a cool box and transferred to the laboratory.

ii. Plant Extraction

The leaves of sage were air-dried, and then powdered using a coffee grinder. Fifty grams of the leaf powder were extracted for three hours in 250 ml of distilled water using the soxhlet apparatus and the source of heating was a warm water bath 45°C. The leaf extract solution was then evaporated at 45°C using a rotary evaporator [11], and the resultant crude extract was used to prepare the required concentrations (250, 500,1000) µg/ml. These concentrations have shown immuno-modulatory activities using *in vitro* mitogenic and comitogenic rat thymocyte tests [12].

iii. Experimental Design

Eight cultures were set-up in triplicates for each subject. Blood cells in culture I was negative control (untreated cells), while in culture II, the cells were treated with the mutagen cytostar (cytarabine: Pharmacia Company, Belgium) at a concentration 125 µg/ml. Cultures III, IV, V were treated with the three concentrations of sage extract respectively, while in cultures VI, VII, VIII, interactions between sage extract (the three concentrations respectively) and cytosar were carried out.

iv. Micronucleus Assay

Two mililiters of RPMI-1640 culture medium (Sigma, USA) were supplemented with (0.1-0.3) ml of PHA (Sigma, USA), and then 0.5 ml of blood was added to the eight culture tubes, together with 0.1 ml (250, 500, 1000) µg/ml of plant extract to cultures III, IV, V, respectively, and cultures VI, VII, VIII, respectively. For cultures II, VI, VII, VIII, 0.1 of cytosar 125 µg/ml was added after a period of 24 hours incubation at 37ºC in an incubator supplemented with 5% CO_2, then the incubation continued up to 72 hours for all cultures. After that, the culture tubes were centrifuged 800 rpm for 5 minutes, and then supernatant was discarded and cell deposit was gently suspended in 5ml of a warm 37°C hypotonic KCl solution 0.1M and 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 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, rinsed with distilled water, and finally air-dried. The slide was examined under oil immersion lens (100X), and the micronucleus index was given as MN per cell [13].

v. Statistical Analysis

Data were given as mean ± standard error (SE), while significant differences between means were assessed by ANOVA and Duncan's tests, in which $P \leq 0.05$ was considered significant using the computer programme SPSS version 11.5.

Results

The results are presented in Table (1), and they are outlined in the following:

i. Culture I (Negative Controls)

The leukemia patients showed a significantly increased frequency of MN formation as compared to healthy controls (0.0236 *vs*. 0.0140 MN/cell).

ii. Culture II (Positive Controls)

The drug cytosar was significantly effective in increasing the MN frequency in healthy controls as compared to negative controls (0.018 *vs*. 0.014 MN/cell). However, such frequency was significantly less than the frequency of micronuclei in cytosar-treated cultures of leukemia patients (0.018 *vs*. 0.023 MN/cell). The latter group showed no significant difference when compared with the corresponding untreated cultures (0.023 *vs*. 0.0236 MN/cell).

iii. Cultures III, IV and V

In healthy controls, the three concentrations (250, 500, 1000) µg/ml of aqueous extract were significantly effective in reducing the spontaneous formation of micronuclei in a concentration-dependent manner (0.0104, 0.0076, 0.0034) MN/cell, respectively as compared to the corresponding untreated cultures (0.014 MN/cell). Blood cultures of leukemia patients treated with the same concentrations of the extract also showed a significant reduced frequency of micronucleus formation (0.0144, 0.0098, 0.0062) MN/cell, respectively as compared to the corresponding untreated cultures (0.0236 MN/cell).

iv. Cultures VI, VII and VIII

In healthy subjects, the first concentration of the extract (250 µg/ml) was not effective in reducing the spontaneous formation of micronuclei as compared to untreated cultures (0.0138 *vs*. 0.014 MN/cell), while the next two concentrations (500, 1000) µg/ml were significantly effective in this regard (0.011, 0.0086) MN/cell, respectively. Similar results were recorded in leukemia patients (0.019, 0.0148, 0.0108, respectively *vs*. 0.0236 MN/cell). However, the latter frequencies were significantly higher than the corresponding frequencies in healthy controls.

Table(1): Micronucleus formation in blood cultures treated with sage leaf aqueous extract of acute lymphoid leukemia patients and healthy controls.

Groups (Cultures)		Concentration (µg/ml)	Mean ± Standard Error (Micronucleus/cell) Healthy Controls (No. = 10)	Leukemia Patients (No. = 10)
Negative Controls (I)		0.0	0.0140 ± 0.0011^{A}	$*0.0236 \pm 0.0023^{A}$
Positive Controls (II)		125	0.0180 ± 0.0013^{B}	$*0.0230 \pm 0.0013^{A}$
Sage Aqueous Extract	III	250	0.0104 ± 0.0002^{C}	$*0.0144 \pm 0.0009^{B}$
	IV	500	0.0076 ± 0.0002^{D}	$*0.0098 \pm 0.0009^{C}$
	V	1000	0.0038 ± 0.0005^{D}	$*0.0062 \pm 0.0004^{D}$
Sage Aqueous Extract +Cytosar	VI	250	0.0138 ± 0.0007^{A}	$*0.0190 \pm 0.0007^{B}$
	VII	500	0.0110 ± 0.0003^{C}	$*0.0148 \pm 0.0009^{B}$
	VIII	1000	0.0086 ± 0.0004^{D}	$*0.0108 \pm 0.0007^{B}$

Different letters: Significant difference ($P \leq 0.05$) between means of the same column.

***Significant difference ($P \leq 0.05$) between means of patients and controls.**

Discussion

The present investigation demonstrated that the sage leaf aqueous extract was significantly effective in reducing the spontaneous or cytosar-induced formation of micronuclei in healthy subjects or all patients; therefore, it is possible to suggest that such extract can be considered as anti-mutagenic agent with the potential to restore the integrity of genetic make-up. Such integrity depends on several repair mechanisms; such as, excision repair, photo-reactivation repair, post-replication repair, error-prone repair and error-free repair, and such mechanisms can be considered as a target for medicinal plants or their active ingredients in terms of enhancement [14]. It has been demonstrated that sage aqueous extract is rich in different chemical ingredients; for instance, flavonoids, phenolic diterpenes, triterpenes, hydroxycinnamic acid derivatives, phenolic glycosides, polysaccharides and essential oils (4), and these constituents can act separately or synergistically in mediating their anti-mutagenic effects. In this regard, it has been found that flavonoids of sage enhances the post-replication repair (5), while others have demonstrated that tannins, flavonoids and terpens stimulates the mechanism of error-free repair. Furthermore, terpens can activate recombinational repair mechanism, beside their action in activating the detoxification enzymes [15]. Beside that, it has also been demonstrated that these compounds stimulate the function of immune system, inhibit the formation of DNA adduct with carcinogens, inhibit hormonal action and metabolic pathways association with the development of cancer and enhance the phase I or II detoxification enzymes. Furthermore, investigations have shown that terpenoids of sage increase tumor latency and decrease tumor multiplicity [16, 17]. Accordingly, the present reduction in MN formation in cultures treated with the aqueous extract may be due to compounds present in the extract that have the ability to inhibit the reactions that induce mutational changes, or induce the activity of enzymes that protect the DNA and induce DNA-repair mechanisms.

The results of micronucleus formation in untreated cultures of all patients revealed that the genetic make-up of patients underwent some alternations (i.e. mutations), and such consequences may lead to establish the leukemia disease, and in this regard, it is always augmented that leukemia requires a mutation(s) before establishing a full-blown disease (10). These genetic alternations can outcome in loss of apoptosis and regulation of cell cycle progression [18]. Both consequences are in favor of establishing a malignant transformation in cells that had these genetic alternations.

In conclusion, the present plant extract can be considered as a potent anti-mutagen, but it is too early to reach such conclusion and further *in vivo* and *in vitro* studies are essential to shed light on its anti-mutagenic potentials. Equally important, a clear chemical analysis of the extract is required.

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