

IN VITRO ANTI-SICKLING ACTIVITY OF ARTEMISIA HERBA-ALBA ASSO (CHIH) METHANOLIC EXTRACT ON SICKLE CELL DISEASE

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ABSTRACT

Background: Sickle cell disease (SCD) is caused by polymerization of abnormal hemoglobin S when oxygen tension decreases. Previous studies have been indicated that some medicinal plants have shown an anti-sickling activity, which indicates a new therapeutic way to a range of people who are affected by this hemoglobinopathy. The current study aimed to assess the *in vitro* anti-sickling activity of *Artemisia herba-alba* Asso methanolic extract.

Methods: The blood samples used in the evaluation of the anti-sickling activity of the plant extract in this study were taken from patients known to have SCD, attending in the King Khaled Hospital in Majmaah. Emmel test was used to assess anti-sickling activity of this plant.

Results: The normal shape of the red blood cells (RBCs) was observed after incubation of RBCs with *A. herba-alba* Asso extract and 2% sodium metabisulfite as compared to control. A significant increase in the percentage of unsickled RBCs was observed after incubation of RBCs with 2% sodium metabisulfite in the presence of 500 and 1000 µg/ml of *A. herba-alba* Asso extract. Besides, the difference between the percentage of unsickled RBCs after 30 and 60 minutes incubation time was significant for 500 µg/ml of *A. herba-alba* Asso extract.

Conclusion: Significant *in vitro* anti-sickling activity of *A. herba-alba* Asso extract was demonstrated in RBCs pretreated with 2% sodium metabisulfite. The results obtained in this study have shown significant *in vitro* anti-sickling activity of *A. herba-alba* Asso extract, and these findings may justify the use of this plant in the management of SCD.

Keywords: *In vitro*, Anti-sickling activity, *Artemisia herba-alba* Asso, Emmel test, Sodium metabisulfite, Percentage, Unsickled red blood cells, Sickle cell disease.

INTRODUCTION

Sickle cell disease (SCD) is an inherited genetic disorder that affects the hemoglobin within the red blood cells (RBCs). The recurrent pain and complications caused by the disease can interfere with many aspects of the patient's life including education, employment, and psychosocial development. The sickle cell trait is now known to be widespread, reaching its highest prevalence in parts of Africa as well as among people in the Mediterranean basin and Saudi Arabia [1].

Artemisia herba-alba Asso (*Asteraceae* family), commonly known as white wormwood or desert wormwood (Arabic name *chih*), is a grayish-strongly aromatic dwarf shrub native to the South Western Europe, Northern Africa, Arabian Peninsula, and Western Asia [2]. The sesquiterpene lactones compounds are the main product which can be obtained from the *A. herba-alba* Asso which give its medical and pharmaceutical important [3]. The pharmacological activities of *A. herba-alba* Asso extract are mentioned by various researchers such as antidiabetic effect [4-7], antimicrobial activity [8], antifungal activities [8,9], and antioxidant effect [9-12].

In this study, we try to find out the anti-sickling effect of methanolic extracts of *A. herba-alba* Asso for reducing complicated management and cost-effective treatment of sickle cell patient.

METHODS**Preparation of methanolic extract of plants**

In this study, *A. herba-alba* Asso (aerial parts) was collected from Al-Qassim region in the North central part of Saudi Arabia in June 2014. A voucher sample is stored at the Department of Medical Laboratories, Majmaah University. The dried plant sample was ground in a blender with a particular size to ensure the powder in identical size, and then

100 g of the powder was soaked for 5-7 days with 1000 ml of 80% methanol at 25°C. After filtration, the filtrate was evaporated with a rotary evaporator to remove the methanol under reduced pressure at 50°C. The dry crude extract of the plant samples was stored in the refrigerator in a dark glass bottle until use. A stock solution 0.1 g/ml from the crude extract was prepared by dissolving 0.1 g of dry crude extract in 1 ml (dimethylsulfoxide [DMSO]) and then diluted in 9 ml normal saline; this stock solution was stored in a refrigerator for 5 days until use.

Collection of blood samples

The blood samples used in the evaluation of the anti-sickling activity of the plant extract in this study were taken from patients known to have SCD, attending in the King Khaled Hospital in Majmaah. All these patients were confirmed regarding their SS status using hemoglobin electrophoresis test. The blood samples were collected in sodium ethylenediamine tetraacetic acid (EDTA) tubes and stored for maximum a few hours for the experiment. A written informed consent was read and signed by all the patients participating in the study. All research procedures have been approved by the National Ethical Committee, King Abdulaziz for Science and Technology, Kingdom of Saudi Arabia, approval number: MUREC-Jan.06/COM-2015.

Anti-sickling activity**Washing of RBCs**

About 4 ml EDTA blood samples obtained from patients were centrifuged at 3000 rpm for 10 minutes to remove the plasma. The resulting packed erythrocytes were washed 3 times with 1 ml sterile normal saline per 5 ml of blood. The samples were then centrifuged each time to remove the supernatant. Washed RBC was then re-suspended in remaining suspension and used for the analysis.

Procedure for anti-sickling activity evaluation

To evaluate the anti-sickling activity of plant extracts, *in vitro* anti-sickling assay was performed: Emmel test (Coutejoie and Hartaing, 1992) as the following: Plant extract a stock solution (10 mg/ml) was prepared by dissolving 0.1 g of dry extract for each plant in 1 ml of 100% DMSO that was prior diluted to 10 ml with normal saline. Then, three diluted solutions in normal saline were prepared from the stock solution of plant extract as follows (250, 500, and 1000 µg/ml).

Washed erythrocyte was mixed with an equivalent volume of 2% sodium metabisulfite ($\text{Na}_2\text{O}_5\text{S}_2$). 10 µl from the above mixture was spotted on a microscope slide then 10 µl from the plant extracts was added and mixed with the blood mixture. 10 µl normal saline was added to one of the slides instead of the plant extract which served as control; all the slides were covered with a cover slip. Paraffin was applied to seal the edges of the cover completely to exclude air (hypoxia), and then, slides were incubated at 37°C for 2-period interval (30 and 60 minutes). Each slide was examined under the oil immersion light microscope, and RBCs were counted in five different fields of view across the slide. The numbers of both sickled and unsickled blood cells were determined, and the percentage of unsickled cells was calculated using the formula:

$$\{(\%) \text{ unsickling} = \text{Number of unsickling cells} \times 100 / \text{total cells}\}$$

All anti-sickling experiments were carried out in triplicate using fresh blood samples. A high power magnification $\times 1000$ was employed to take representative images from different fields to display morphological changes of RBCs during different stages of the experiment using a digital camera.

Statistical analysis

All data were reported as the mean \pm standard deviation; statistical analysis was performed using SPSS Statistics 17. A paired t-test is used to find the significance of the difference between the means of the two groups (control vs. test samples). $p \leq 0.05$ considered significant.

RESULTS AND DISCUSSION

Extractive yield

The extractive yield of the studied plant was 11.7%.

Anti-sickling activity of methanolic extract of *A. herba-alba* Asso

Effect of plant crude extracts on sickle cell morphology

Fig. 1 shows the morphology of RBCs after incubation of RBCs with 2% sodium metabisulfite in the presence of 0.9% NaCl (control). Figs. 2-4 show morphology of RBCs after incubation of RBCs with 2% sodium metabisulfite in the presence of 250, 500, and 1000 µg/ml of crude extract of *A. herba-alba* Asso.

As shown in Fig. 1, almost all RBCs were sickle shape which confirmed the nature of sickle red cells which have property to change their normal shape (biconcave shape) to sickling shape under hypoxic condition.

Fig. 2 shows that few RBCs retained their normal biconcave shape while the rest transfigured to sickle shape.

Figs. 3 and 4 show that almost all RBCs retained their biconcavity revealing the anti-sickling activity of methanolic extract of *A. herba-alba* Asso. This finding points toward anti-sickling activity of the crude methanolic extract of this plant under hypoxic condition.

The morphology of sickle RBC in the presence of a methanolic extract of *A. herba-alba* Asso (Figs. 3 and 4), compared to the control (Fig. 1), showed that the majority of the sickle RBC reversed their shapes to the normal biconcave shape. This confirms the activity of the methanolic extract of *A. herba-alba* Asso on the normalization of the erythrocytes form. The same results were observed for the anthocyanins extracts from several plants used in traditional medicine in D.R. Congo against sickle cell anemia [13-18].

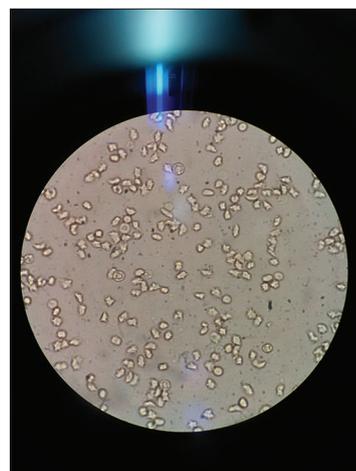


Fig. 1: Morphology of sickle red blood cells: Untreated or control (NaCl 0.9%; $\text{Na}_2\text{O}_5\text{S}_2$ 2%)

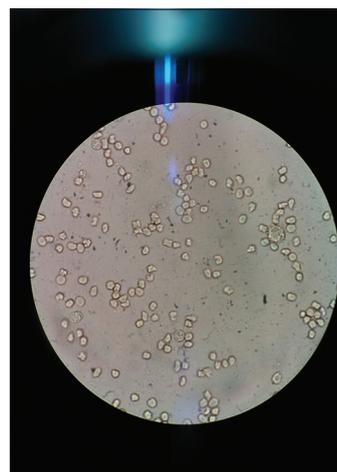


Fig. 2: Morphology of sickle red blood cells: Treated with 250 µg/ml of methanolic extract of *Artemisia herba-alba* Asso, [NaCl 0.9%; $\text{Na}_2\text{O}_5\text{S}_2$ 2%]

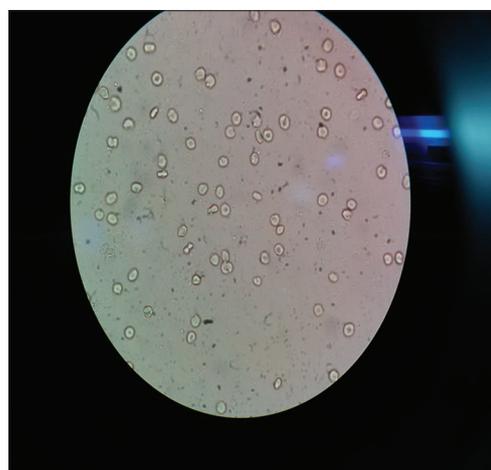


Fig. 3: Morphology of sickle red blood cells: Treated with 500 µg/ml of methanolic extract of *Artemisia herba-alba* Asso, [NaCl 0.9%; $\text{Na}_2\text{O}_5\text{S}_2$ 2%]

This finding also agrees with results of others who found that the majority of sickle-shaped erythrocytes are reversed into normal and

Table 1: Anti-sickling activities of methanolic extract of *A. herba-alba* Asso and normal saline as control

Time of incubation (min)	Percentage of unsickled RBCs			
	RBCs+Na ₂ O ₅ S ₂ +250 µg/ml	RBCs+Na ₂ O ₅ S ₂ +500 µg/ml	RBCs+Na ₂ O ₅ S ₂ +1000 µg/ml	RBC+Na ₂ O ₅ S ₂ +normal saline (control)
30	15.9±4.4	60.3±6.5	87.9±3.2	12.9±0.46
p	0.2995	0.0002	0.0001	
60	17.9±1.0	85.3±2.2	91.0±4.2	17.1±10.0
p	0.9018	0.0003	0.0003	

Each value represents the mean value±SD, (n=3), p≤0.05 considered significant, compared to control. RBC: Red blood cell, SD: Standard deviation, *A. herba-alba*: *Artemisia herba-alba*

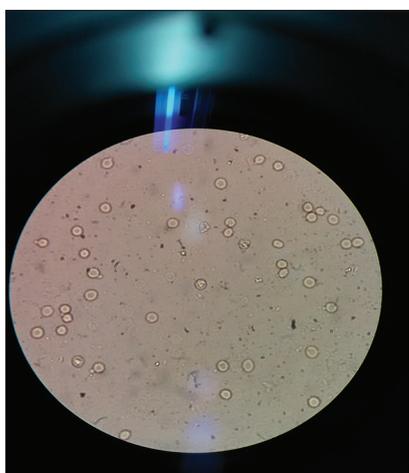


Fig. 4: Morphology of sickle red blood cells: Treated with 1000 µg/ml of methanolic extract of *Artemisia herba-alba* Asso (NaCl 0.9%; Na₂O₅S₂ 2%)

biconcave shape when sickle erythrocytes mixed with the aqueous extracts of *Justicia* species, the aqueous extract of *Ocimum basilicum* L., aqueous extracts of *Dicliptera colorata* C. B. Clarke, *Euphorbia hirta* L., and *Sorghum bicolor* (L.), and methanolic extract of *Zingiber officinale* Roscoe [13,18-20].

Effect of methanolic extract of *A. herba-alba* Asso on the percentage of unsickled RBCs

Table 1 shows the percentage of unsickled RBCs after incubation RBCs of sickle cells disease patients with 2% sodium metabisulfite in the presence of 250, 500, and 1000 µg/ml of methanolic extracts of *A. herba-alba* Asso at two different incubation time (30 and 60 minutes). As shown in Table 1, compared to control the highest percentage of unsickled RBCs at 30 minutes, incubation time was observed for 1000 µg/ml of methanolic extract of *A. herba-alba* Asso (87.9%) followed by 500 µg/ml of methanolic extract of *A. herba-alba* Asso (60.3%) and 250 µg/ml of methanolic extract of *A. herba-alba* Asso being the lowest (15.9%) and the highest percentage of unsickled RBCs at 60 minutes incubation time was observed for 1000 µg/ml of methanolic extract of *A. herba-alba* (91.0%) followed by 500 µg/ml of methanolic extract of *A. herba-alba* Asso (85.3%) and 250 µg/ml of methanolic extract of *A. herba-alba* Asso being the lowest (17.9%). A significant increase in the percentage of un-sickled RBCs was observed at concentrations of 500 µg/ml and 1000 µg/ml of methanolic extract of *A. herba-alba* Asso in both incubation times (30 and 60 minutes).

These findings coincide with others who found that the normalization rate of the form of the sickle cells were 87% for *Justicia gendarussa*, 92% for *Justicia insularis*, and 80% for *Justicia tenella*, the aqueous extract of *Adansonia digitata* showed an anti-sickling activity with a maximal normalization rate of 65.7%, the aqueous extract of *O. basilicum* L. showed an anti-sickling activity with a normalization rate of 87.00%, and the aqueous extract of *E. hirta* L. showed an anti-sickling activity

with a normalization rate of 70%. The methanolic extract of *Z. officinale* Roscoe showed an anti-sickling activity with a percentage of unsickled RBCs of 99.2% [13,16,18-20].

Effect of incubation time on the percentage of unsickled RBCs

As shown in Table 1, after 30 minutes incubation time of RBCs of sickle cells disease patients with 2% sodium metabisulfite in the presence of 250, 500, and 1000 µg/ml of methanolic extract of *A. herba-alba* Asso, the percentages of unsickled RBCs were 15.9, 60.3, and 87.9, respectively, whereas after 60 minutes incubation time with same concentration the percentages of unsickled RBCs were 17.9, 85.3, and 91.0, respectively; moreover, there is a significant difference between the percentage of unsickled RBCs after 30 and 60 minutes incubation time of RBCs of sickle cells disease patients with 2% sodium metabisulfite in the presence of 500 µg/ml of methanolic extract of *A. herba-alba* Asso (p=0.01).

CONCLUSION

The results obtained in this study have shown significant *in vitro* anti-sickling activity of *A. herba-alba* Asso extract, and these findings may justify the use of this plant in the management of SCD.

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