



## ***In vitro* Antisickling Activity of *Achillea fragrantissima* (Forssk) Sch. Bip (Qaysūm) Methanolic Extract on Sickle Cell Disease**

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### ABSTRACT

Sickle cell disease (SCD) is caused by polymerization of abnormal haemoglobin S when oxygen tension decreases. This lead to changes in the shape of red blood cells and anemia. Previous studies have been indicated that some medicinal plants have shown an antisickling 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* antisickling activity of *Achillea fragrantissima* (Forssk) Sch. Bip methanolic extract. Emmel test was used to assess antisickling activity of this Plant. The normal shape of the red blood cells was not observed after incubation of red blood cells with *Achillea fragrantissima* (Forssk) Sch. Bip methanolic extract and 2% sodium metabisulfite as compared to control. No significant increase in the percentage of unsickled red blood cells was observed after incubation of red blood cells with 2% sodium metabisulfite in the presence of 250µg/ml, 500µg/ml and 1000µg/ml of *Achillea fragrantissima* (Forssk) Sch. Bip methanolic extract. In conclusion No significant *in vitro* antisickling activity of *Achillea fragrantissima* (Forssk) Sch. Bip methanolic extract was demonstrated in red blood cells pretreated with 2% sodium metabisulfite.

**Keywords:** *In vitro*; Antisickling activity; *Achillea fragrantissima* (Forssk) Sch. Bip; Emmel test; SCD.

### INTRODUCTION

Sickle-cell disease (SCD) is an inherited genetic disorder that affects the haemoglobin within the red blood cells. 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.<sup>1</sup>

*Achillea fragrantissima* (Forssk) Sch. Bip is a wild herbaceous shrub belongs to the Asteracea family.<sup>2</sup> It has a different common names according to the country found in such as Lavender cotton (English); Guarda roba (French); Qaysūm (Arabic).<sup>3</sup> It has been used for many years in traditional medicine in Middle Eastern countries for the treatment of respiratory diseases, skin diseases, gastro- intestinal disturbances, high blood pressure, stomach aches and diabetes.<sup>4,5</sup> Recent reports demonstrated anti-inflammatory, antioxidant and antiproliferative capacities of *A. fragrantissima* extracts.<sup>6,7,8</sup>

The pharmacological effects of *Achillea fragrantissima* (Forsk.) Sch. Bip are Antioxidative effects.<sup>8</sup> Lacked any antirheumatic or antiinflammatory effects in carrageenan-induced acute inflammation in rats<sup>9</sup>, but exerted antimicrobial and antiviral activities.<sup>10,11</sup> Modulatory effects on rat ileum muscle contraction<sup>12</sup>. Beneficial in preventing/treating neurodegenerative diseases.<sup>6</sup> Aqueous extract exhibited strong cytotoxicity and larvicidal activities.<sup>13,14</sup> Aqueous and hydro-alcoholic extracts of *Achillea fragrantissima* (Forsk.) (Asteraceae)

grown in Jordan were screened for their antioxidant, antimicrobial, antiplatelet, anti-proliferative and acetylcholinesterase (AChE) inhibition efficacy.<sup>15</sup>

In this study we try to find out the antisickling effect of methanolic extracts of *Achillea fragrantissima* (Forssk) Sch. Bip. for reducing complicated management and cost effective treatment of sickle cell patient.

### MATERIALS AND METHODS

#### Preparation of Methanolic Extract of Plants

In this study, *Achillea fragrantissima* (Forsk.) Sch. Bip (aerial parts) was collected from Al-Qassim region, Saudi Arabia in June 2014. A voucher sample is stored at the Department of Medical laboratories, AL-majmaa University. The dried plant sample were ground in a blender with a particular size to ensure the powder in identical size, and then 100g of the powder was soaked for 5-7 days with 1000ml 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 were stored in refrigerator in dark glass bottle until use. A stock solution 0.1g/ml from the crude extract was prepared by dissolving 0.1g of dry crude extract in 1ml (DMSO) and then diluted in 9ml normal saline, this stock solution was stored in 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 sickle cell disease, attending in the King Khaled Hospital in Majmaah. All



these patients were confirmed regarding their SS status using haemoglobin electrophoresis test. The blood samples were collected in sodium 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 & Technology, Kingdom of Saudi Arabia, approval number: MUREC-Jan.06/COM-2015.

### Antisickling Activity

#### Washing of RBCs

Four milliliters EDTA blood samples obtained from patients were centrifuged at 3,000 rpm for 10 min to remove the plasma.

The resulting packed erythrocytes were washed 3 times with 1ml sterile normal saline per 5ml of blood.

The samples were then centrifuged each time to remove the supernatant. Washed RBC were then re-suspended in remaining suspension and used for the analysis.

#### Procedure for antisickling activity evaluation

In order to evaluate the antisickling activity of our plant extracts, in vitro antisickling assay was performed: Emmel test (Coutejoie and Hartaing, 1992) as the following:

Plant extract a stock solution (10mg/ml) was prepared by dissolving 0.1 g of dry extract for each plant in 1ml of 100% dimethylsulfoxide (DMSO) that was prior diluted to 10ml with normal saline.

Then three diluted solutions in Normal saline were prepared from the stock solution of plant extract as follows (250µg/ml, 500µg/ml and 1000µg/ml).

Washed erythrocyte were mixed with an equivalent volume of 2% sodium metabisulfite ( $\text{Na}_2\text{O}_5\text{S}_2$ ).

10µl from the above mixture was spotted on a microscope slide then 10µl from the plant extracts was added and mixed with the blood mixture.

10µl 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 min and 60 min). Each slide was examined under the oil immersion light microscope and red blood cells were counted in five different fields of view across the slide.

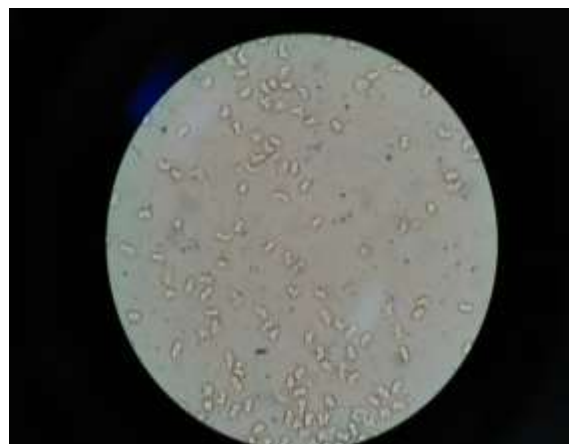
The numbers of both sickled and unsickled blood cells were determined and the percentage of unsickled cells were calculated using the formula:  $\{(\% \text{ unsickling} = \text{Number of unsickling cells} \times 100 / \text{total cells})\}$ . All anti-sickling experiments were carried out in triplicate using a fresh blood samples. A high power magnification

X1000 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  $\pm$  SD., 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 value  $\leq$  0.05 considered significant.

## RESULTS AND DISCUSSION



**Figure 1:** Morphology Of sickle RBCs: untreated or control, [NaCl 0.9%;  $\text{Na}_2\text{S}_2\text{O}_5$  2%]

### Extractive Yield

The extractive yield of studied plant was 9.7%.

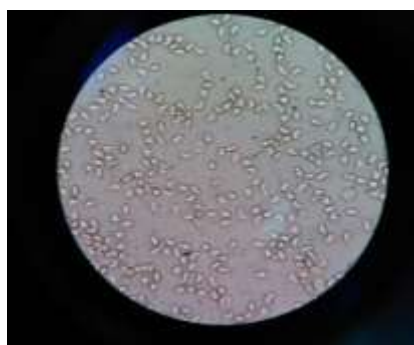
### Antisickling Activity of Methanolic Extract of *Achillea fragrantissima* (Forssk) Sch. Bip

#### Effect of plant crude extracts on sickle cell morphology

Fig. 1 shows Morphology of red blood cells after incubation of red blood cells with 2% sodium metabisulfite in the presence of 0.9% NaCl (control), Fig. 2, 3, and 4 show morphology of red blood cells after incubation of red blood cells with 2% sodium metabisulfite in the presence of 250µg/ml, 500µg/ml and 1000µg/ml of crude extract of *Achillea fragrantissima* (Forssk) Sch. Bip.

As shown in Fig. 1 almost all red blood cells 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, 3, and 4 show that almost all red blood cells were sickle-shape. This finding indicating that the crude methanolic extract of this plant had no antisickling activity under hypoxic condition, a finding disagrees with results of previous similar studies.<sup>16-20</sup>



**Figure 2:** Morphology of sickle RBCs: treated with 250µg/ml of methanolic extract of *Achillea fragrantissima* (Forssk) Sch. Bip, [NaCl 0.9%; Na2s205 2%]



**Figure 3:** Morphology of sickle RBCs: treated with 500µg/ml of methanolic extract of *Achillea fragrantissima* (Forssk) Sch. Bip, [NaCl 0.9%; Na2s205 2%]



**Figure 4:** Morphology of sickle RBCs: treated with 1000µg/ml of methanolic extract of *Achillea fragrantissima* (Forssk) Sch. Bip, [NaCl 0.9%; Na2s205 2%]

**Effect of methanolic extract of *Achillea fragrantissima* (Forssk) Sch. Bip on the percentage of unsickled red blood cells**

Table 1 shows the percentage of unsickled red blood cells after incubation red blood cells of sickle cells disease patients with 2 % sodium metabisulfite in the presence of 250µg/ml, 500µg/ml and 1000µg/ml of methanolic extracts of at two different incubation times (30 min and 60 min).

As shown in Table 1, compared to control the percentage of unsickled red blood cells at 30 min incubation time was observed for 1000µg/ml (17.26%) followed by 500µg/ml (10.26%), then 250µg/ml (5.89%) and the percentage of unsickled red blood cells at 60 min incubation time was observed for 1000µg/ml (17.61%) followed by 500µg/ml (8.61%), then 250µg/ml (6.40%). No significant differences in the percentage of un-sickled RBCs was observed at concentrations of 250µg/ml, 500µg/ml and 1000µg/ml of methanolic extract of *Achillea fragrantissima* (Forssk) Sch. Bip in both incubation times (30min and 60 min).

These findings disagree with previous studies such as<sup>16,18,20</sup>

**Effect of incubation time on the percentage of unsickled red blood cells**

As shown in Table 1, after 30 min incubation time of red blood cells of sickle cells disease patients with 2 % sodium metabisulfite in the presence of 250µg/ml, 500µg/ml and 1000µg/ml of methanolic extract of *Achillea fragrantissima* (Forssk) Sch. Bip the percentages of unsickled red blood cells were 5.89, 10.26 and 17.26, respectively while after 60 min incubation time with same concentration the percentages of unsickled red blood cells were 6.40, 8.61 and 17.61 respectively, moreover there is no significant difference between the percentage of unsickled red blood cells after 30 min and 60 min incubation times.

**Table 1:** Antisickling activities of methanolic extract of *Achillea fragrantissima* (Forssk) Sch. Bip and normal saline as control. Each value represents the mean value ± S.D., (n =3), P value ≤ 0.05 considered significant, compared to control

Time of incubation (min)	% of unsickled red blood cells			
	Red blood Cells + Na <sub>2</sub> O <sub>5</sub> S <sub>2</sub> +250 µg/ml	Red blood cells + Na <sub>2</sub> O <sub>5</sub> S <sub>2</sub> + 500µg/ml	Red blood cells + Na <sub>2</sub> O <sub>5</sub> S <sub>2</sub> + 1000 µg/ml	Red blood cell+ Na <sub>2</sub> O <sub>5</sub> S <sub>2</sub> + normal saline (Control)
30 min	5.89±2.13	10.26±2.89	17.26±8.87	5.65± 2.07
P value	0.8922	0.1115	0.1772	
60	6.40±1.34	8.61±0.48	17.61±4.45	7.10±0.61
P value	0.4602	0.1223	0.0664	

**CONCLUSION**

The results obtained in this paper have shown No significant *in vitro* anti-sickling activity of *Achillea fragrantissima* (Forssk) Sch. Bip extract. Further studies need to confirm these findings.

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