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ANTISICKLING PROPERTIES OF *AZADIRACHTA INDICA* A. JUSS LEAVES, FRUITS, AND STEM EXTRACTS

Devshree Verma, Meena Sahu and K. K. Harris*

Department of Zoology, Government DB Girls' Postgraduate College, Raipur (C.G).

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***Corresponding Author**
K. K. Harris
Department of Zoology, Government DB Girls' Postgraduate College, Raipur (C.G).

ABSTRACT

Antisickling activities of *Azadirachta indica* leaves, fruits and stem extracts showed the presence of phenols, alkaloids, flavonoids, saponins and tannins suggesting its medicinal properties, making it widely used in traditional medicines. The inhibitory activities for different concentrations of extracts revealed maximum inhibition activity (IA) of 72.64% in leaves in a concentration of 10 mg/ml; fruit showed a IA of 70.02% in a concentration of 10.00 mg/ml; while the stems showed a IA of 72.70% in a concentration of 0.5mg/ml and the reversal of sickled Erythrocyte for different concentrations of extracts revealed maximum reversal activity (RA) of 73.49% in leaves in a concentration of 5.0 mg/ml; fruit showed a RA of 72.39% in a concentration of 5.0 mg/ml; while the stems showed a RA of 68.04% in a concentration of 1.5 mg/ml. Presence of these compounds justifies the anti-sickling properties possessed by this plant. In the light of the results of phytochemical analysis of the leaves, fruits and stem of *Azadirachta indica* A. JUSS we may conclude that the antisickling properties possessed by the plant is due to the underlying phytochemicals present in the plants. Further research towards formulation of an effective remedy, containing phytochemicals from plants possessing antisickling properties, in various concentrations, for the relief of over 270 million Global Sickle Cell Disease patients during the Crisis Stage of the disease needs to be initiated.

KEYWORDS:- *Azadirachta indica*, Phytochemistry, Antisickling activity, Sickle Cell Disease, Raipur, Chhattisgarh.

INTRODUCTION

We have recognized plants for their several lifesaving and therapeutic properties since ancient times.[1] Lifestyle and eating habits alterations among the people make it vital to refer to herbal medicine as an alternative or complementary therapeutic measure. About 70% of the Worlds' population depends entirely upon such traditional medical therapies as their primary form of health care.[2,3] Phytochemicals in the plant extracts possess therapeutic properties and are used in traditional practice by the traditional healers. Active principle is a substance found in medicinal plants, containing the healing property of plants.[4] This active principle differs from plant to plant and their examples incorporates: anthraquinones, flavonoids, glycosides, saponins, tannins etc. Plants also contain other compounds such as morphine, atropine, codeine, steroids, lactones and volatile oils, which possess medical values to treat different diseases. In recent years, these active principles have been extracted and used in different forms such as infusions, syrups, concoctions, decoctions, infused oils, essential oils, ointments and creams.[5,6]

MATERIALS AND METHODS

I. Phytochemical analysis

i. Collection of plant samples

The fresh leaves, fruits and stems *of Azadirachta indica* A JUSS were collected simultaneously from Raipur and from cultivated farms and the open fields of Mahasamund district.

ii. Qualitative phytochemical analysis

a. Tannins

0.5 g of the extract was dissolved in 10 ml of distilled water, then a few drops of 1% ferric chloride solution was added to obtain a brownish green or blue-black precipitate, which confirms the presence of tannin.

b. Saponins

0.5 g of the extract was dissolved in 5 ml distilled water. The mixture was shaken vigorously. Formation of stable persistent froth shows the presence of saponins. A further addition of 6 drops of olive oil while shaking forms an emulsion, confirming the presence of Saponins.

c. Reducing sugars

1 gm of the extract was dissolved in 10 ml of distilled water. This extract was boiled with Fehling solution A and B in test tube and colour changes were observed. Presence of brick red colour indicated the presence of reducing sugar.

d. Alkaloids

6 ml of extract was mixed with 6 ml of 1% HCl in steam bath, then it was filtered. 1 ml of Mayer's reagent was added. Presence of turbidity shows presence of alkaloids. Further addition of a few drops of olive oil to form an emulsion confirmed the presence of alkaloids.

e. Terpenoids

0.5 gm extract was dissolved in 2 ml of chloroform then 3 ml concentrated sulfuric acid was added, a reddish brown colour in interphase indicates the presence of terpenoids.

f. Flavonoids

5 ml dilute ammonia was added to 5 ml extract and then 5 ml concentrated sulfuric acid was added. Formation of yellow colour shows the presence of flavonoids.

g. Cardiac glycosides

2.5 g of extract was added to 2.5 ml distilled water. 1 ml glacial acetic acid containing a few drops of ferric chloride was added then 0.5 ml of concentrated sulfuric acid was added. Presence of brown ring at the interphase indicates the presence of deoxy sugar. A violet ring below the brown ring was observed, while a greenish ring also appears above the brown ring, confirming the presence of Cardiac Glycosides.

h. Anthraquinones

2.5 g extract was dissolved in 5 ml of conc. Sulfuric acid and filtered. The filtrate was dissolved in 2.5 ml of chloroform. Chloroform layer was pipetted into a tube and 0.5 ml of 10% diluted ammonia was added. Formation of pink red or violet colour shows the presence of anthraquinones.

i. Phenols

2 ml of extract was dissolved in 4 ml of distilled water and added few drops of 10% FeCl3. Appearance of blue or green colour indicates presence of phenols.

II. Antisickling assay

i. Preparation of plant extracts

We used four solvents for the extraction of different parts of the plants based on their increasing polarity. These are ethanol, methanol and chloroform and petroleum ether. 30g of the powdered leaves, Fruits and stems of *Azadirachta indica* A JUSS were extracted with different solvents in Soxhlet apparatus in 250 ml of each solvents separately for 48 hours and they were concentrated by slow evaporation process.[7] The obtained crude extracts were kept in a closed container for preliminary qualitative phytochemical analysis. Aqueous- methanol (60-80^{o}C) in 1:3 as solvents.[8] The prepared extracts were stored at 4^{o}C in freeze in dried form and used for the antisickling activity test. Varying concentrations have been prepared from the dried extracts and used for the antisickling assay was varied from 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0 and 10.0 mg/ml of leaves, fruit and stems of *Azadirachta indica* A JUSS.

ii. Collection of blood sample

The blood samples used in the evaluation of the antisickling activity of plants in this study were collected from electrophoresis confirmed HbSS SCD patients belonging to the age-group 16 to 25 years, of both sexes and it was ensured that they do not take any Allopathic or Ayurvedic medications. The samples were drawn in the presence of a qualified pathologist as approved by the Institutional Ethical Committee, Govt' DB Girls' PG College, Raipur.

A quantity of 5.0 ml of fresh blood samples were collected each time by way of vein-puncture in EDTA (Ethylene di-amine tetra acetic acid) anticoagulant tubes and mixed gently to prevent lysing of the red blood cells. The study was approved by the Institutional Ethical Committee, Govt' DB Girls' PG College, Raipur. Blood samples were stored in ± 4^{o}C in refrigerator each time.

iii. Sickling Reversal & Inhibition assays

For inhibitory sickling activity test 0.2 ml HbSS sample was used. 0.2 ml of phosphate buffered saline solution and 0.2 ml of different concentration of the extracts were added serially. Sealed with liquid paraffin wax and incubated in a thermo-stated water bath at 37^{o}C for about 4 hours. After incubation 0.6 ml of freshly prepared 2% sodium meta-bi-sulphite solution was added. These mixtures were again incubated for about one and half hours in 37^{o}C in a thermo-stated water bath. After incubation the liquid paraffin wax was removed with the help of a Pasteur pipette and the resultant mixture was fixed in 3 ml of 5% v/v buffered formalin.[9]

iv. Counting of cells

The fixed cells were centrifuged and the supernatants were decanted with a capillary tube. Slides were prepared from fixed cells after the process of centrifugation. One or two drops was applied on a microscopic slide and carefully covered with a cover slip and sealed with wax. Prepared slides were observed under a high-power objective (x40 and x 100) of Research Trinocular Microscope (LABOMED VISION 2000) About four hundred (400) cells (both sickle and normal erythrocytes) were counted and the percentage sickled cells were recorded.[9]

RESULTS AND DISCUSSION

A. Phytochemical analysis

Phytochemical estimation

The results of qualitative phytochemical estimation of tannin, alkaloids, reducing sugar, saponin, terpenoids, flavonoids, cardiac glycosides, anthraquinones and phenols in the leaves, fruits and stem of *Azadirachta indica* A. JUSS are presented in Table-1. The phytochemical tests were performed on the ethanolic, methanolic, chloroform and petroleum ether extracts of the leaf, fruit and stem of the *Azadirachta indica* A. JUSS. Tannins were present in methanolic extracts of all the components viz., leaves, fruits and stem; while in the ethanolic, extracts only leaves and stems were found positive. However, the chloroform and petroleum ether extracts showed negative results in all the components studied. Alkaloids were present in ethanolic and methanolic extracts of all the components viz., leaves, fruits and stem; while in the chloroform extracts only the leaves and fruits showed positive results. However, in the present in ethanolic, methanolic, chloroform extracts of fruits were positive. Reducing sugars were present in ethanolic, methanolic, chloroform extracts of all the components viz., leaves, fruits and stem; while in the petroleum ether extract only the fruits were positive. Saponins were present in ethanolic, methanolic, chloroform extracts only leaves and stem were positive; while in the petroleum ether extract only the leaves were positive; Terpenoids were present in ethanolic, methanolic, chloroform and petroleum ether extracts of all the components viz., leaves, fruits and stem; Flavonoids were present in ethanolic, methanolic, chloroform extracts of all the components viz., leaves, fruits and stem; while in the petroleum ether extract only the leaves and fruits were positive; Cardiac glycosides were present in ethanolic extract only stem while in the chloroform and petroleum ether extract only stem were positive; Anthraquinones were present in chloroform extract only stem were positive However, the petroleum ether extract only the fruits were positive; Phenols were present

ethanolic and methanolic extracts of all the components viz., leaves, fruits and stem; However, the chloroform and petroleum ether extract only leaves were positive.

Table 1: Phytochemical constituents of different extracts of the leaves, fruits and stems of *Azadirachta indica* A. JUSS.

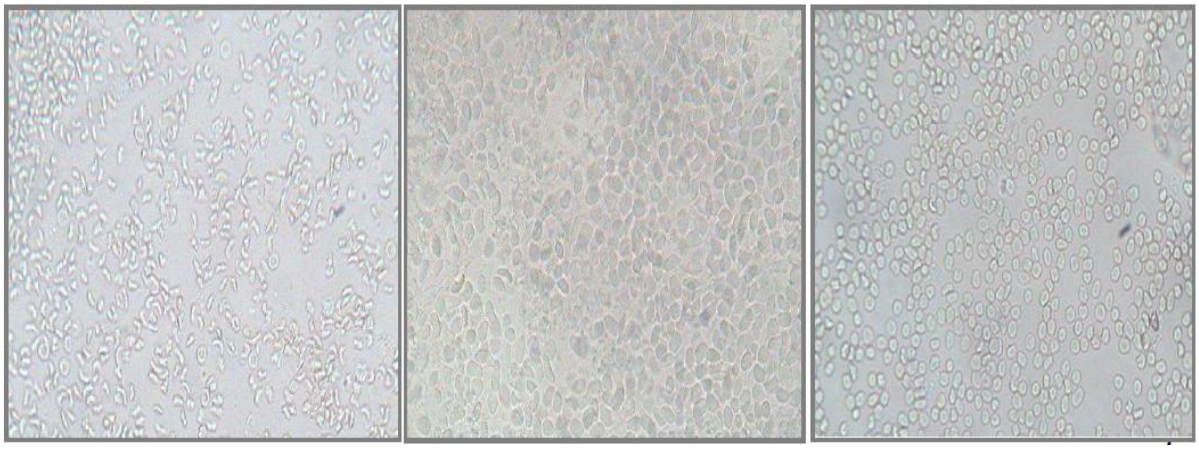
Sr. no	Components	Ethanolic *			Methanolic			Chloroform			Petroleum ether		
		1#	2	3	1	2	3	1	2	3	1	2	3
1	Tanins	+	-	+	+	+	+	-	-	-	-	-	-
2	Alkaloids	+	+	+	+	+	+	+	+	-	-	+	-
3	Reducing sugars	+	+	+	+	+	+	++	++	+	-	+	-
4	Saponins	+	-	+	+	-	+	+	-	+	+	-	-
5	Terpinoids	+	+	+	+	+	+	+	+	+	+	+	+
6	Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+
7	C glycosides	-	-	+	-	-	-	-	-	+	-	-	+
8	Anthroquinon	-	-	-	-	-	-	-	-	+	-	+	-
9	Phenols	+	+	+	+	+	+	+	-	-	+	-	-

EXTRACTS; #: 1-leaf ;2-fruits;3– stem; + (Positive); - (Negative)

B. Antisickling inhibition assay

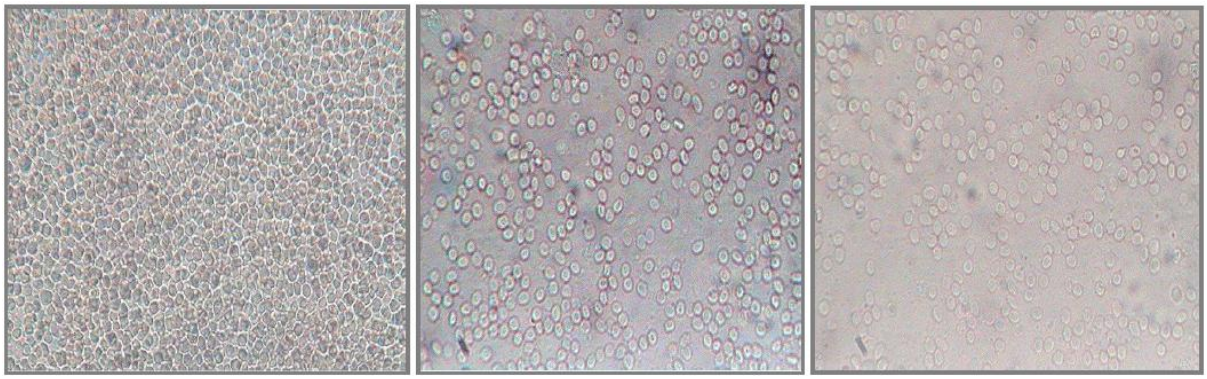
Antisickling activity Inhibition of erythrocyte activity of Azadirachta indica A. JUSS.

The inhibitory activities found in increasing percentage in Leaves, fruits and Stems (Fig.-1). The inhibitory activities for different concentrations of extracts revealed maximum inhibition activity (IA) of 72.64% in leaves in a concentration of 10 mg/ml; fruit showed a IA of 70.02% in a concentration of 10.00 mg/ml; while the stems showed a IA of 72.70% in a concentration of 0.5mg/ml. Morphology of drepanocytes (Sickle cells) of the HbSS blood of non-treated, treated with PBS (-ve control), PHBA (+ve control) and different concentration of the extracts of the leaves, fruit and stem extract of *Azadirachta indica* A.JUSS. Were presented on (Plate- 2&3). The morphology of drepanocytes of the HbSS blood (a) nontreated; (b) treated with Phosphate buffer saline (PBS Negative control); (c) treated with Para hydroxy benzoic acid (PHB as Positive control) and morphology of drepanocytes of the HbSS blood treated with *Azadirachta indica* A. JUSS. shows inhibition effect on Leaf extract (a) (10. 0 mg/ml) Fruit extract (b) (10. 0 mg/ml) stem extract (c) (0.5 mg/ml) extract concentration is given below.



A B C

Plate 1: Morphology of drepanocytes of the HbSS blood (a) nontreated; (b) treated with Phosphate buffer saline (PBSNegative control); (c) treated with Para hydroxy benzoic acid (PHB as Positive control).



A B C

Plate 2: Morphology of drepanocytes of the HbSS blood treated with *Azadirachta indica* A. JUSS. Shows inhibition effect on Leaf extract (a) (10. 0 mg/ml) Fruit extract (b) (10. 0 mg/ml) stem extract (c) (0.5 mg/ml) extract concentration.

Duncan multiple range for inhibitory activity

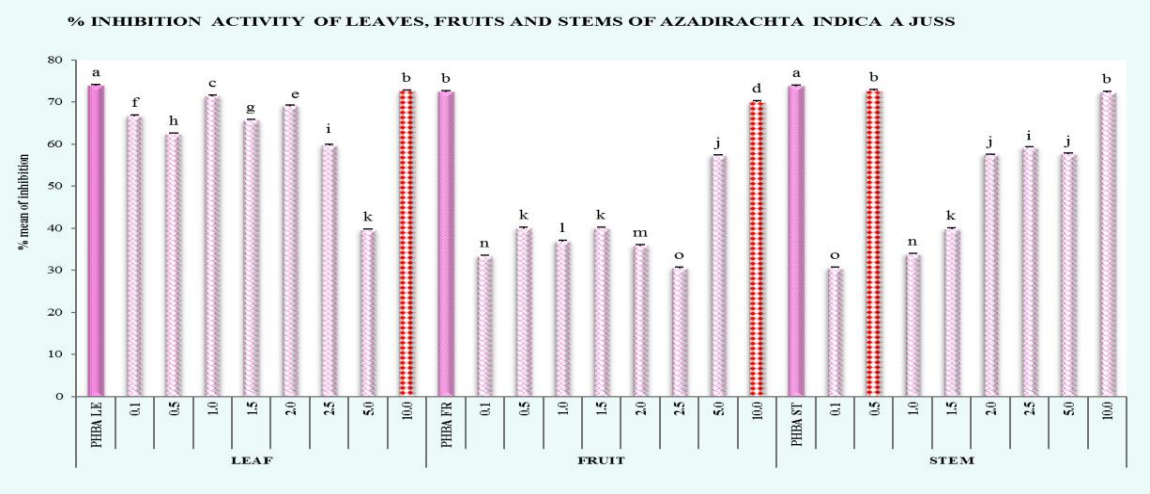


Figure 1: Difference in mean inhibition of sickle cell *in vitro* at different concentrations of leaves, fruit and stems extracts of *Azadirachta indica* A. JUSS. Means (± SE) having similar alphabets are not statistically significant from each other at p < 0.05 (Based on Duncan's multiple-range test).

C. Antisickling reversal assay

Reversal of Sickled Erythrocyte activity of Azadirachta indica A. JUSS.

The reversal activities found an increasing percentage in Leaves, fruits and Stems (Fig-2). The Reversal of sickled Erythrocyte for different concentrations of extracts revealed maximum reversal activity (RA) of 73.49% in leaves in a concentration of 5.0 mg/ml; fruit showed a RA of 72.39% in a concentration of 5.0 mg/ml; while the stems showed a RA of 68.04% in a concentration of 1.5 mg/ml. Morphology of drepanocytes (Sickle cells)of the HbSS blood treated with different concentrations of the extracts of the leaves, fruit and stem extract of *Azadirachta indica* A. JUSS are depicted in Plate-3.

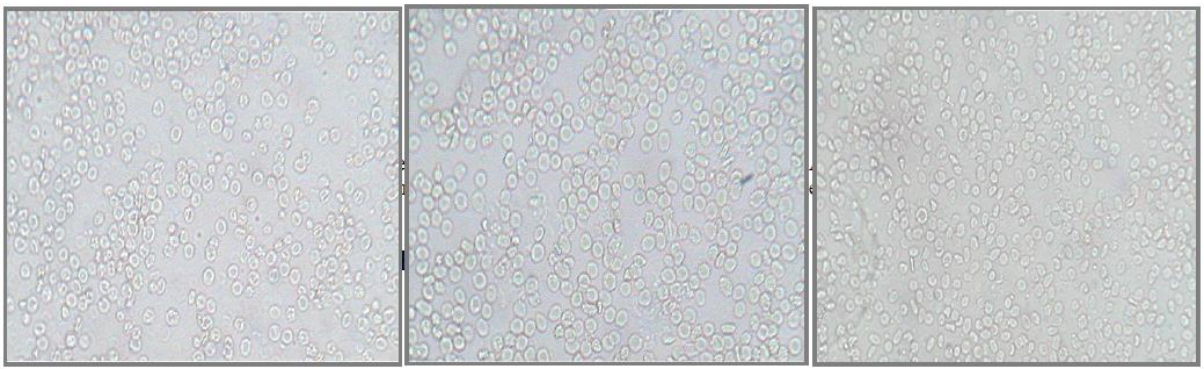


Plate 3: Morphology of drepanocytes of the HbSS blood treated with *Azadirachta indica* A.JUSS. Shows reversal effect on Leaf extract (a)(5.0 mg/ml) fruit extract (b)(5.0 mg/ml) stem extract (c) (1.5 mg/ml) extract concentration.

Duncan multiple range for reversal activity

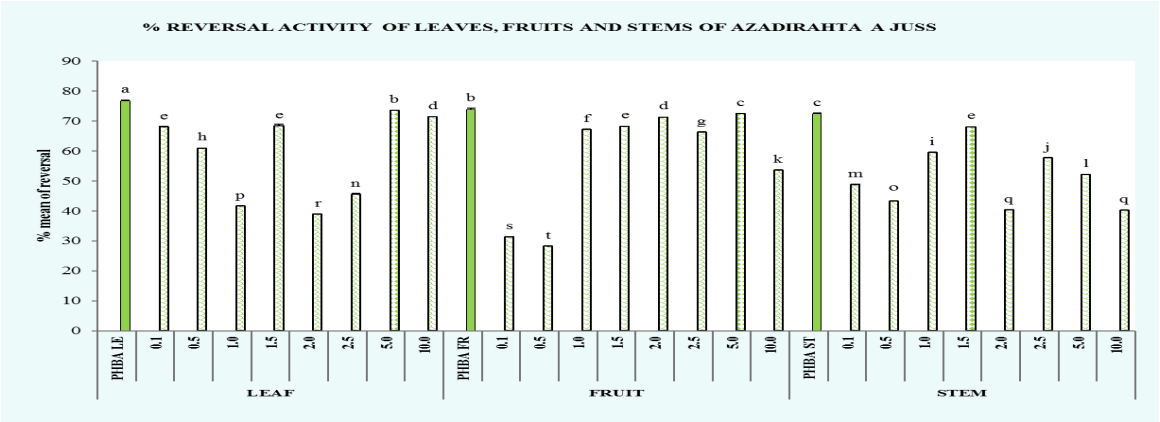


Figure 2: Difference in mean reversal of sickle cell *in vitro* at different concentrations of leaves, fruits and stems extracts of *Azadirachta indica* A JUSS Means (± SE) having similar alphabets are not statistically significant from each other at p < 0.05 (Based on Duncan's multiple-range test).

The developing world phytomedicines could be important in the management of SCD, some of these plants reported are *M. charantia*,[10] *Cymbopogon citratus* and *Camellia sinensis*,[11] *Scoparia dulcis*,[12] *aged garlic*[13] studied that crude aqueous extract of *Zanthoxylum macrophylla* roots possessed anti-sickling properties.[14] studied that Methanol and Chloroform extracts *Carica papaya* L fruit pulp possessed anti-sickling properties.[15] The aqueous and methanol extracts of *C. papaya* leaves possessed Antisickling activity,[16] studied that methanol extracts and the aqueous fractions of the leaf, seed and seed pod of *G. kola* possessed anti-sickling properties,[17] studied that methanolic extract bark of *Morus alba* L. found anti-sickling activity,[18] The antisickling activity of four cucurbits species (*T. Occidentalis, C. maxima, C. sativus* and *C. lanatus*,[19] *O. basilicum* leaves extract show antisickling activity;[20] aqueous extract of *Carica papaya* leaves show antisickling potential (Naiho et al. 2015),[21] studied *C. cajan* leaves and seed, *Z. zanthoxyloides* leaves and *C. papaya* leaves. Have antisickling properties,[22] studied *Combretum glutinosum* leaves have antisickling properties,[23] *S. setigera* methanolic leaves extract show antisickling activity,[24] roots and stem barks fractions of *Newbouldia laevis* P. Beauv. Possessed anti-sickling properties,[25] mineral composition of some plants (*Annona senegalensis* Pers., *Alchornea cordifolia* (Schumach. & Thonn.) Müll. Arg. and *Vigna unguiculata* (L.) Walp. possessed anti-sickling properties.[26]

CONCLUSIONS

The results of the present study revealed the presence of alkaloids, flavonoids and tannins in various proportions in different parts of *A. indica* extracts. These phytochemicals are reported by various workers to possess anti sickling propensities and in recent years these bioactive components have been used in different forms such as infusions, syrups, concoctions, decoctions, essential oils, ointments and creams. Hence, *Azadirachta indica* A. JUSS can well be used alone or in combination with other plant extracts for formulating a potent remedy for the management of sickle cell disease.

The findings of Antisickling assays performed in blood samples obtained from Sickle Cell Disease patients and in the light of the results of phytochemical analysis of the leaves, fruits

and stem of the plant studied, *Azadirachta indica* A. JUSS we may conclude that the antisickling properties possessed by the plant is due to the underlying phytochemicals present in the plants. Further research to characterize and separate the phytochemicals responsible for antisickling effects possessed by the plant, should be commenced in order to attain drug discovery and formulation of an effective remedy, containing phytochemicals from the plant, in various concentrations, for the relief of over 270 million Global Sickle Cell Disease patients during the ―Crisis Stage of SCD.

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REFERENCES

1. Olagunjua JA, Adeneyeb AA, Fagbohunkac BS, Bisugac NA. Nephroprotective activities of the aqueous seed extract of *Carica papaya* Linn. In carbon tetrachloride induced renal injured Wistar rats: a dose- and time-dependent study. *Biology and Medicine*, 2009; 1(1): 11-19.
2. Sarkadi B, Tosteson D, Giebisch G, Tosteson D. In Membrane Transport in Biology, Springer and Heidelberg, 1979; 2: 117−160.
3. Bewaji CO, Olorunsogoand OO, Bababumi EA. Sickle cell membrane-bound (Ca2+ and Mg2+)- atpase: Activation by 3,4-dihydro-2, 2-dimethyl-2H-1-1-benzopyran-6-butyric acid. A novel antisickling agent, Cell Calcium, 1985; 6: 237-244.
4. Ballas SK. and Marcolina, M.J. Hyperhemolysis during the evolution of uncomplicated acute painful episodes in patients with sickle cell anemia. Transfusion, 2006; 46(1): 105-110.
5. Walters MC, Storband R, Patience M. Impact of bone marrow transplantation for symptomatic sickle cell disease: aninterim report. Blood, 2000; 95: 1918-24.
6. Sofowora A, Medicinal plants and traditional medicine in Africa. Spectrum Books. Ibadan, 1993; 289: 10-15.
7. Harbone, J.B. Phytochemical methods: Chapman and Hall Ltd. London, 1973; 49-188.

8. Ogoda OJ, Akubue PI, Okide GB. The Kinetics of Reversal of Pre-sickled Erythrocytes by the aqueous extract of *Cajanus cajan* seeds. Phytotherapy Research, 2002; 16: 1-3.
9. Cyril-Olutayo CM, Anthony EA, Alani DM. Antisickling properties of the fermented Mixture of *Carica papaya* Linn and *Sorghum bicolor* (L.) Moench. AJPP, 2009; 3(4): 140-143.
10. Semiz, A. and A. Sen. Antioxidant and chemo protective properties of *Momordica charantia* L. (bitter melon) fruit extract. AJB, 2007; 6: 27 3-277.
11. Ojo OO, Kabutu FR, Bello M, Babayo U. Inhibition of paracetamol-induced oxidative stress in rats by extracts of lemongrass (*Cymbopogon citratus*) and green tea (*Camellia sinensis*) in Rats. AJB, 2006; 5(12): 1227-1232.
12. Adaikpoh MA, Orhue NEJ, Igbe I. The protective role of *Scoparia dulcis* on tissue Antioxidant defense system of rats exposed to cadmium. AJB, 2007; 6(10): 1192-1196.
13. Ohnishi ST, Ohnishi T, Ogunmola. GB. Green tea extract and aged garlic extract inhibitanion transport and sickle cell dehydration in vitro. Blood Cells, Molecules and Diseases, 2001; 27: 148-157.
14. Elekwa I, Monanu MO, Anosike EO In vitro effects of aqueous extracts of *Zanthxylum Macrophyla* roots on adenosine triphosphatases from human erythrocytes of different genotypes. Biokemistri, 2005; 17(1): 19-25.
15. Mojisola OC, Adebolu EA, Durosinmi Muheez Alani DM. Antisickling Properties of *Carica papaya* Linn. J N Prod, 2008; 1: 56-66.
16. Imaga NOA, Gbenle GO, Okochi VI, Akanbi1 SO, Edeoghon SO, Oigbochie V, Kehinde MO and Bamiro SB. Antisickling property of *Carica papaya* leaf extract. AJBR, 2009; 3(4): 102-106.
17. Adejumo OE, Ayoola MD, Kolapo AL, Orimoyegun, VO, Olatunji PO. Antisickling activities of extracts of leaf, seed and seed pod of *Garcinia kola* Heckel. AJPP, 2011; 5(1): 48-52.
18. Meselhy KM, Hammad LN, Farag N. Novel Antisickling, Antioxidant and Cytotoxic Prenylated Flavonoids from the Bark of Morus alba L. Life Sci J, 2012; 9(3).
19. Nwaoguikpe RN, Ujowundu CO, Okwu GN. The Antisickling Potentials of Four Curcubits (*T. Occidentalis, C. Maxima; C. Sativus and C. Lonatus*) Sch. J. App. Med. Sci, 2013; 1(3): 191-198.
20. Tshilanda DD, Mpiana PT, Onyamboko DNV, Mbala BM, Ngbolua KN, Tshibangu DST, Bokolo MK, Taba KM, Kasonga TK. Antisickling activity of butyl stearate isolated from *Ocimum basilicum* (Lamiaceae). Asian Pac J Trop Biomed, 2014; 4(5): 393-398.

21. Naiho AO, Okonkwor BC. Okoukwu C. Anti-Sickling and Membrane Stabilizing Effects of *Carica papaya* Leaf Extract. BJMMR, 2015; 6(5): 484-492, 2015.224.
22. Ismaila O, Nurain CO, Bewaji, Johnson JS, Davenport RD, Zhang Y, Potential of Three Ethnomedicinal Plants as Antisickling Agents. Mol. Pharmaceutics, 2017; 14: 172−182.
23. Sall C, Ndoye SF, Dioum MD, Seck I, Gueye RS, Faye B, Thiam CO, Seck M, Gueye PM, Fall D, Fall M, Ndiaye T. Phytochemical Screening, Evaluation of Antioxidant and Anti-sickling Activities of Two Polar Extracts of *Combretum glutinosum* Leaves. Perr. Ex DC. BJAST, 2017; 19(3): 1-11.
24. Abdullahi B. In Vitro Anti-Sickling Effect of Crude and Partially Purified Fractions of Methanolic Extract of *Steculia Setigera* Leaf on Human Sickled Red Blood Cells. SWJ, 2018; 13(4): 1597-6343.
25. Dermane A, Kpegba K, Metowogo K, Joppa MK, Aklikokou AK. Antisickling activity evaluation of fraction obtained from whole extracts of *Newbouldia laevis* P. BEAUV (BIGNONIACEAE), 2019; 11(2): 0975-1491.
26. Kitadi JM, Inkoto CL, Lengbiye EM. Tshibangu DST, Tshilanda DD, Ngbolua KN, Taba KM, Mbala BM, Schmitz B and Mpiana PT. Mineral Content and Antisickling Activity of *Annona senegalensis*, *Alchornea cordifolia* and *Vigna unguiculata* Used in the Management of Sickle Cell Disease in the Kwilu Province (Congo, DR) IBRR, 2020; 11(3): 18-27.