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ORIGINAL ARTICLE

Anti sickling potential and chemical profiling of traditionally used *Woodfordia fruticosa* (L.) Kurz leaves

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Abstract *Woodfordia fruticosa* (L.) Kurz is a widely used plant in traditional medicine systems. The tribal communities of Amarkantak, Madhya Pradesh (India) are using this plant for the treatment of general weakness, blood related complications like blood deficiency, blood purification and for the treatment of symptoms related to sickle cell disease (SCD). SCD is a genetic disease with life threatening complications. In the absence of any drugs without any side effects, the alternative plant based therapies that may either reduce/ reverse the sickling of the red blood cells can be safe and effective therapeutic agents. We evaluated *W. fruticosa* extracts for phytoconstituents, anti-oxidant and anti-inflammatory properties. Anti-sickling properties of the extracts were evaluated by estimation of reverse sickling, polymerization inhibition and osmotic fragility assays. Chemical profiling of the methanol extract was done using LC-MS analysis. Phytochemicals such as alkaloids, steroids, tannin, and saponins were present in all the extracts. Methanol extract displayed maximum reversal (66 ± 1%) of sickled Red blood Cells (RBC) and significantly inhibited Hb polymerization. The hexane and methanol extracts led to minimum hemolysis of sickled RBC in the osmotic fragility assays. Total tannin (365 ± 2.4 TAE) content was highest in acetone extract, while the total flavonoid and phenolic content (156.9 ± 2.0 QE) and (113.7 ± 0.7 GAE) were highest in methanol extract. The methanol extract displayed minimum IC_{50} (8.1 ± 1.5) in 2, 2-diphenylpicrylhydrazyl (DPPH) while the acetone extract had minimum IC_{50} (215.8 ± 5.7) in 2,2′-azino-bis 3-ethylbenzo thiazoline-6-sulfonic acid (ABTS) assay. The hexane extract displayed maximum Ferric reducing anti-oxidant power (FRAP) value (1.5 ± 0.5 mM Fe(II)/mg dry weight) that were higher than methanol and aqueous extracts (1.45 ± 0.1, 1.45 ± 0.05 mM Fe(II)/mg dry weight). The methanol extract provided maximum RBC protection from hemolysis (73.8 ± 0.8%). Maximum Lipoxygenase (LOX) inhibition was observed by the acetone and methanol extracts at 400 μg/mL while

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the hexane extract displayed maximum Xanthine oxidase (XO) inhibition (57.0 ± 0.5%). LC-MS profiling of the methanol extract identified several secondary metabolites that might be responsible for the observed activities. The results validate the traditional use of *W. fruticosa* and present us with potential compounds for further development of novel anti sickling agents.

1. Introduction

Sickle cell disease (SCD) is the most common inherited blood disorder and a "molecular disease" arising due to the substitution of valine for glutamic acid at position 6 of the β-globin chain of hemoglobin (Hb) (Strader et al., 2020). Clinical manifestations of SCD are mainly characterized by chronic hemolysis and acute vaso-occlusive crisis, which are responsible for severe acute and chronic organ damage. SCD is widespread in sub-Saharan Africa, in the Middle East, Indian subcontinent, and some Mediterranean regions. The World Health Organization recognized hemoglobinopathies, including SCD, as a global public health problem and urged national health systems worldwide to design and establish programs for the prevention and management of SCD. In India, SCD is predominantly prevalent amongst scheduled tribes (ST) and scheduled caste (SC) populations that are the most socioeconomically disadvantaged subgroups in the country. The pathophysiology associated with SCD arises due to the polymerization of the resulting sickle haemoglobin variant (HbS). Individuals with SCD experience considerable morbidity and in the absence of any effective treatment, the most severe cases can be fatal within the first few years of life (Hockham et al., 2018). Being an inherited blood disorder, there is no single cure for the treatment of SCD. The treatment varies from person to person depending on the age, symptoms, severity. A drug hydroxyurea (HU) is recommended that can reduce the number of painful episodes, reduce hospital stays and the requirement of blood transfusions but is known to cause neutropenia, thrombocytopenia or worsening anemia. Patients may also experience nail or skin changes most often involving hyperpigmentation. The side effects of the drug during pregnancy or for longer durations are not completely known. Some new medications such as Endari (L-glutamine oral powder) and Oxbryta (voxelotor) have been approved by the Food and Drug Administration (FDA) but are not cost effective. Stem cell transplant can actually cure the SCD but require a matched donor (Sickle Cell Disease, 2021). Due to the complexity and multiple pathophysiological targets, development of low cost drugs with novel mechanism of action is urgently required. The new drug development strategies can be divided into two broad categories namely pathophysiology-related that includes development of agents which reduce/ prevent sickling, agents targeting SCD vasculopathy and sickle cell-endothelial adhesive events and anti-oxidant molecules. Natural products obtained from medicinal plants and used in traditional medicines are of great importance for the development of modern medicine. Several treatment methods have been historically developed to cure a variety of ailments and life threatening conditions. The formulations of the traditional medicine are mostly derived from the medicinal plants identified over a period of time with respect to mode of preparation, plant/ plant part selection, and the suitable time for collection. This knowledge need to be documented and preserved as it can assist in the development of modern drugs in the areas of lead compound identification, examination of drug-like activity and exploring physicochemical, biochemical, pharmacokinetic, and toxicological characteristics leading to significant cost reductions. A lot of biologically active compounds from higher plants have been developed as modern drugs that are playing a vital role in providing relief from pain and diseases (Yuan et al., 2016). A number of plants including *Cajanus cajan* seeds, *Zanthoxyllum zanthoxyloides* root, *Carica papaya* unripe fruit and leaves, *Parquetina nigrescens, Plumbago zeylanica, Uvaria chamae, Elaesis guineensis, Boerhavia diffusa* are reported to have antisickling properties and are extensively used by the traditional healers for treating sickle cell crises associated morbidity among the under privileged classes of the society (Adejumo et al., 2010; Ogwutum et al., 2018; Shah et al., 2020). *W. fruticosa* is one such plant used by the tribal healers of the Amarkantak region, India for the treatment of SCD and associated pathophysiology. It has been reported that the leaves of *W. fruticosa* are used for treating fever, haemoptysis, rheumatism, ulcers and for milk enhancement in livestock (Birajdar et al., 2014). The plant also finds extensive use in both the Ayurvedic and Unani systems of medicine for the treatment of a variety of diseases. Extracts and metabolites of this plant, particularly those from flowers and leaves have been reported to possess pharmacological activities such as anti-tumor, anti-microbial and anti-ulcer (Das et al., 2007; Kumar et al., 2016). An herbal composition containing *W. fruticosa* has been patented for the management of gynecological disorders (Katiyar et al., 2002). The current study is an attempt towards exploring natural therapeutics. Based on the traditional knowledge of the tribal communities of Amarkantak, Madhya Pradesh, India, *W. fruticosa* has been selected for evaluating its potential in reverse sickling, inhibition of polymerization and osmotic fragility, inflammation inhibition and membrane stabilization.

2. Material and methodology

2.1. Collection and authentication of *W. fruticosa*

W. fruticosa Kurz (syn. Woodfordia floribunda Salisb.) belonging to the family Lythraceae, is an evergreen tropical shrub with 5–7 m height. It is well branched with a reddish-brown bark (Fig. 1). *W. fruticosa* is locally known as *birul*, *phoolchuhi* (Grover et al., 2013; Khan et al., 2019). In the months of April and May, the fresh leaves of *W. fruticosa* were collected from the Amarkantak, a hill station in the Pushparajgarh tehsil (22°44′ latitude north and 81°44′ longitude east and

Fig. 1 *W. fruticosa* Leaves (A) and Flowers (B).

altitude 1060.70 m) Madhya Pradesh, India. Collected plant parts were identified and authenticated by the Department of Botany, Indira Gandhi National Tribal University Amarkantak MP India and a voucher specimen has been stored at the Herbarium of Department of Botany, IGNTU (Voucher number IGNTU19/BIOT/WF).

2.2. Extract preparation

The collected leaves were dried at room temperature and grounded into a fine powder. Extracts were prepared using a soxhlet apparatus. In the first cycle 100 mL of hexane was used for 5–7 consecutive cycles. This procedure was repeated successively using acetone, chloroform, methanol and water. The extracts were filtered using Whatman No.1 filter paper and solvents were removed through evaporation under reduced pressure at 45 °C using a rotary evaporator (Huie 2002; Zygmunt et al., 2003).

2.3. Preliminary phytoconstituents screening

The chemotropic variation the chemical composition of the plant varies with the season, geological region, nutrient contents in soil, environment, growing conditions, growth stages etc. Therefore it is imperative to identify all the quantitative phytochemicals present in any plants under study. The presence of alkaloids, tannins, saponins, flavonoids, glycosides, phenols and steroid in the extracts was analyzed as mentioned elsewhere (Harborne 1998; Senguttuvan et al., 2014) with slight modifications.

2.4. Anti-sickling activity

2.4.1. Human blood samples

Blood from SCD patients was collected by visiting the Primary Health Centre, Amarkantak, District Anuppur, Madhya Pradesh (India). The ethical approval for the research work was obtained from the IGNTU-Institutional Human Ethics Committee (Ref: IEC No. 003/IGNTU-IEC/2019–01) dated 16–02-19.

2.4.2. Reverse sickling assay

The reverse sickling assay was performed at different concentrations of the *W. fruticosa* extracts (250, 500 and 1000 µg/mL). Freshly collected blood was washed twice in five volumes of phosphate buffer saline (PBS, pH 7.4) by centrifugation at 3000 revolutions per minute (RPM) for 5 min (min). Cell suspension (100 µL) was aliquoted in different tubes for control, standard and test samples. 100 µL of freshly prepared 2% sodium metabisulfite was added to these tubes containing 100 µL of washed blood cells. After mixing the tubes were incubated for 2 h (h) at 37 °C. 100 µL of PBS (control), standard HU and plant extract (250, 500 and 1000 µg/mL) were added to the individual tubes that were further incubated for 2 h at 37 °C. Reaction mixture (10 µL) was taken and used for visualization under the microscope (EVOS XL Cell imaging system, Thermo Fischer Scientific) at 100x magnification. Cells were counted with the help of haemocytometer (Nurain et al., 2017). The assay was performed in triplicate.

The percentage of sickled cells was calculated as follows:

Percent sickling = (Number of sickled cells/total number of counted cells) × 100

Percent reverse sickling = (100 − Percent sickling).

2.4.3. Polymerization inhibition assay

Freshly prepared 2% sodium metabisulfite was used for inducing the partially sickled RBC. of sodium metabisulfite (880 µL) was mixed with 20 µL of pre-washed blood followed by addition of 100 µL PBS (control) / standard (HU)/ plant extracts (250, 500, 1000 µg/mL). The absorbance was taken immediately at 700 nm at every 2 min time interval for 30 min (UV-1800 24 V Shimadzu, Japan). Assay was performed in triplicates as reported earlier (Nwaoguikpe et al., 2013; Nurain et al., 2017). The rate of polymerization was calculated using the following formula:

Rate of polymerization = [(Final absorbance − Initial absorbance)/ 30] × 100.

2.4.4. Osmotic Fragility assay

NaCl solution in varying concentrations (0–0.9%) was prepared. To 3 mL of NaCl solution taken individually, 50 µL

of prewashed blood was added followed by addition of 100 µL of PBS (control)/ standard (HU)/ plant extract (1 mg/mL) to the different tubes. Mixture was incubated at 37 °C for 4 h. The tube without NaCl was used as control while the tube having 0.9 % NaCl was labeled as blank respectively. After incubation, the tubes were centrifuged at 3000 RPM for 5 min. The supernatant was transferred to the cuvette and the absorbance was taken at 540 nm (UV-1800 24 V Shimadzu, Japan). Assay was performed in triplicates (Pauline et al., 2013; Nurain et al., 2017).

Percent of hemolysis was calculated as: (Absorbance of the supernatant in test sample / Absorbance of the supernatant without NaCl) × 100

2.5. Anti-Inflammation assays

2.5.1. Membrane stabilization assay

A mixture of PBS (1 mL), 50 mM hypo saline (2 mL) was taken in five different tubes followed by addition of 0.5 mL of RBC suspension to respective tubes. To these tubes 1 mL of different plant extracts/standard (200, 400, 600, 800, 1000 µg/mL) were added and mixed. The tubes were incubated for 30 min at 37 °C. The tubes were centrifuged for 20 min at 3000 RPM. The supernatant was collected in different tubes and the absorbance was measured at 560 nm using UV spectrophotometer (UV-1800 24 V, Shimadzu, Japan) to estimate the hemoglobin content (Bolajoko et al., 2015). The percentage of hemolysis was estimated as:

Hemolysis (%) = (Absorbance of test /Absorbance of control) X 100

The percentage of human RBC membrane stabilization or protection was calculated by the formula: % Protection = (100 – % Hemolysis)

2.5.2. Lipoxygenase inhibition assay

For determination of lipoxygenase (LOX) inhibition activity, a lipoxygenase inhibitor screening kit (760700, Cayman Chemical Company, USA) was used as per the manufacturer's protocol. All required material buffers and standard was provided in the kit. Different concentration of the plant extract were prepared 100, 200, 300, 400 µg/mL in dimethyl sulfoxide (DMSO). Absorbance was taken at 590 nm using an ELISA plate reader. The percent inhibition was calculated as follows:

$$Inhibition\ (\%) = \frac{(Absorbance\ Control - Absorbance\ Sample) \times 100}{(Absorbance\ Control)}$$

2.5.3. Xanthine oxidase inhibition assay

Xanthine oxidase (XO) inhibitory activity of the extracts with xanthine as substrate was performed as described. Varying dilutions of each extract (100, 200, 300, 400, 500 µg/mL were prepared in phosphate buffer from the stock solution. To the assay mixture that consisted of 1 mL sodium phosphate buffer (0.15 mM, pH 7.5) and 1 mL 0.15 mM xanthine (prepared in phosphate buffer), 100 µL plant extract/ standard were added followed by incubation of 10 min at 37 °C. After incubation 100 µL (0.1 U/mL in phosphate buffer) of XO solution (Himedia, RM7620) was added to the reaction mixture and the tubes were incubated for another 30 min at 37 °C. Allopurinol (prepared in DMSO) was used as a standard. The reaction was stopped by addition of 1 mL 1 N HCl. The absorbance was measured at 290 nm (UV-1800 24 V, Shimadzu, Japan). The percentage of XO inhibitory activity was evaluated by estimation of the uric acid formation in control as compared to that formed in the samples (Wahyuningsih et al., 2016). The XO inhibition percentage was calculated as:

$$XO\ inhibition(\%) = \frac{(Absorbance\ Control - Absorbance\ Sample) \times 100}{(Absorbance\ Control)}$$

2.6. Anti-Oxidant assays

2.6.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH is purple colored, free and stable radical used to evaluate the scavenging activity. DPPH solution and test samples were prepared in methanol. To 100 µL of 0.01% DPPH solution, 100 µL of plant extract/ standard (5, 10, 50, 100 µg/mL) were added and the plates were incubated in the dark for 30 min at RT. Absorbance was read at 517 nm (Multiskan ELISA reader, Thermo Scientific). Gallic acid was used as standard and DPPH alone was used as blank (Chang et al., 2001; Boly et al., 2016). The IC_{50} value (µg/mL) values were calculated using linear regression analysis**.** Percentage of the radical scavenging activity (RSA) was calculated as:

$$RSA(\%) = \frac{(Absorbance\ Control - -Absorbance\ Sample) \times 100}{(Absorbance\ Control)}$$

2.6.2. Ferric reducing anti-oxidant power (FRAP) assay

FRAP reagent was prepared immediately before the start of the experiment by mixing acetate buffer, 2, 4, 6-tripyridyl-Striazine (TPTZ) and $FeCl_3{\cdot}6H_2O$ in 10:1:1 Reduction of ferric to ferrous ion was measured as reducing power of the plant extract. Ascorbic acid was used as standard. The electron donating capability of different plant extracts was measured using previous methods (Gan et al. 2010). FRAP assay utilizes antioxidants as reductants in a redox–linked colorimetric assay employing easily reduced oxidant, Fe III. Reduction of a ferric tripyridyltrazine to Ferrous II can be monitored by the measurement of the absorbance at 593 nm. To 2 mL of -FRAP reagent, 1 mL of plant extracts were added at varying concentrations (100, 200, 300, 400, 500 µg/mL). The reaction mixture was incubated in dark for 30 min before taking the absorbance at 593 nm (UV-1800 24 V Shimadzu, Japan). Reducing power of plant extract and standard was calculated using regression analysis [x = (y-0.1522)/1.375] of the standard calibration curve of ferrous sulphate prepared with concentrations ranging from 0.2, 0.4, 0.6, 0.8, 1.0 mM (Al-Abd et al., 2015).

2.6.3. 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay

ABTS solution (14 mM) was prepared and mixed with potassium per sulfate (4.95 mM) (1:1 v/v) and left for overnight incubation at RT in the dark to yield a dark colored solution containing ABTS radical cations. The prepared ABTS solution was diluted with distilled water till the absorbance reached 0.70 ± 0.02 at 745 nm at 30 °C. To 300 µL of standard (ascorbic acid)/ plant extracts (100, 200, 300, 400, 500 µg/mL) 3.0 mL of ABTS working standard were added individually and mixed. The decrease in absorbance of tested samples was mea-

sured at 745 nm after five min of incubation (Re R et al., 1999). The scavenging effect was calculated as:

$$\frac{(Absorbance\ Control\ --\ Absorbance\ Sample\ ) \times 100}{(Absorbance\ Control)}$$

The antioxidant capacity of the plant extracts was expressed in terms of IC_{50} (Inhibitory concentration) values.

2.6.4. Total phenolic content (TPC)

Folin-Ciocalteu (FC) method was utilized for the determination of total phenolic content of *W. fruticosa* extracts. To 100 µL of FC reagent 1.16 mL of distilled water was mixed followed by addition of 20 µL of standard gallic acid at different concentrations (100, 200, 300, 400, 500 µg/mL) in individual tubes. To the mixture 300 µL of 20 % Na_2CO_3 solution was added. For the test samples, 20 µL of different plant extracts (1 mg/mL) were added to the sample tubes. The reaction tubes were incubated for 30 min at 40 °C followed by measurement of the absorbance at 760 nm. Phenolic content of plant extracts sample was expressed as mg gallic acid equivalent/g dry material (Arabshahi-Delouee et al., 2007, El Jemli et al., 2016).

2.6.5. Total flavonoid content (TFC)

For determining the total flavonoid content in different extracts of *W. fruticosa,* the aluminum chloride colorimetric method was used (Quettier et al., 2000; Hossain et al., 2011). Quercetin at different concentrations (100, 200, 300, 400 and 500 µg/mL) was used for preparing the calibration curve. 0.6 mL of quercetin solutions or plant extracts were separately mixed with 0.6 mL of 2% aluminum chloride and mixed. The mixture was incubated for 15 min at RT. The absorbance of the reaction mixtures was measured against blank at 440 nm wavelength (UV–Vis spectrophotometer, Shimadzu). The concentration of total flavonoid content in the test samples was calculated from the calibration plot ($y = 0.0043x + 0.1585$; $R^2 = 0.9972$) and expressed as mg quercetin equivalent (QE)/g of dried plant material. All the experiments were carried out in triplicate.

2.6.6. Total tannin content (TTC)

Estimation of total tannin content in the different leaf extracts of *W. fruticosa* was performed as described previously. For preparing the standard curve, 100 µL of tannic acid at different concentrations (100, 200, 300, 400, 500 µg/mL) was used and the volume of tube was made upto 7 mL with distilled water. To these tubes 20 mM ferric chloride and 8 mM potassium ferric cyanide (prepared in 0.1 M HCl) were added and the contents were mixed. The absorbance of the samples was taken at 700 nm. In the similar fashion, 100 µL of different plant extracts (1 mg/mL) were taken instead of standard and the measurements were done. The tannin content was expressed as mg of tannin per gram dry weight of extract (Muthukumaran et al., 2016).

3. Chemical profiling of the *W. fruticosa* methanol extract.

3.1. LC-MS analysis

Based on the bioactivities performed, the methanol extract was selected for LC-MS analysis out of the five tested extracts. LC-MS analysis was performed using Waters ACQUITY QSM with column ACCUCORE C18 (150 X 2.1, 2.6um). The final method was also transferred to a Waters ACQUITY QSM Quantum Ultra LC-MS/MS system, that involved a Waters ACQUITY QSM binary pump (SN- L10QSM943A), a Waters ACQUITY FTN autosampler {Software Version: 1.50.1481 Firmware Version: 1.50.317 (Jul 11, 2011) SN: M10SDI443M}. Four solvents (solvents A, B, C and D) were mixed by the binary pump and used as the mobile phase. The LC parameters have been presented in **Supplementary** Table 1**.** The accurate mass data of the molecular ions were processed through the software Version: 1.50.1481 (Waters technology) and they included extracted ion chromatograms, and elemental compositions. All analytes were identified using their elemental composition, accurate mass measurement, elution order, and comparison with reliable data in compounds database. Based on the specific retention time (RT) in HPLC, the different peaks in the chromatogram were compared with those already reported in the literature and used to identify the compounds present in the *W. fruticosa* methanol extract.

4. Statistical analysis

The data obtained was expressed as mean ± standard error of the three determinations. Data was analyzed using one-way analysis of variance (ANOVA), with the help of statistical software PRISM 5.01. The criterion for statistical significance between the results of experimental and control groups was follows: $p \leq 0.05$ was considered significant and $p > 0.05$ considered as non-significant, whereas $p \leq 0.01$ and $p \leq 0.001$ were considered highly significant and very highly significant respectively.

5. Results

5.1. Evaluation of preliminary qualitative phytoconstituents

The phytochemical screening of *W. fruticosa* leaf extracts was performed for analysis of alkaloids, flavonoids, saponins, tannins, glycosides, phenols, and steroids in different extracts. The hexane extract displayed the presence of alkaloids, phenols and steroids. The chloroform extract displayed the presence of alkaloids, saponins, glycosides, phenols and steroids. Flavonoids, saponins, tannins, phenols and steroids were present in the aqueous extract. Methanolic and acetone extract were rich in alkaloids, flavonoids, tannins, glycosides, phenols and steroids (Table 1).

5.2. Evaluation of anti-sickling properties in different *W. fruticosa* leaf extracts

5.2.1. Reverse sickling assay

Blood obtained from SCD patients was incubated with *W. fruticosa* plant extracts at three different concentrations. Microscopic observation for physiological changes in terms of reverse sickling was captured (**Supplementary** Fig. 1). The study demonstrated that methanolic extract was capable of reverse sickling in a concentration dependent manner. Approximately 66 ± 1% of the RBC was found to be reversed sickled at 1000 µg/mL. The reverse sickling in aqueous and hexane

Table 1 Qualitative phytochemical analysis of various leaf extracts of *W. fruticosa*.

Plant constituents	WFH	WFA	WFC	WFM	WFW
Alkaloid	+	+	+	+	–
Flavonoids	–	+	–	+	+
Saponins	–	–	+	–	+
Tannins	–	+	–	+	+
Glycosides	–	+	+	+	–
Phenols	+	+	+	+	+
Steroids	+	+	+	+	+

'+': Present, '-': Absent. WFH (Hexane extract), WFA (Acetone extract), WFC (Chloroform extract), WFM (Methanol extract), WFW (Aqueous extract).

extract was 47.5 ± 0.5% and 42.7 ± 0.7% respectively while in the standard drug HU it was found to be 49.5 ± 0.5%. Chloroform and acetone extract displayed least reversal in sickling (Table 2).

5.2.2. Polymerization inhibition assay

Point mutation in β-globin chain of Hb leads to hydrophobic binding amongst the modified Hb molecules in the hypoxic conditions. The rate of polymerization inhibition was calculated for different extracts. The results clearly depict the significant rate of polymerization inhibition in the methanolic extract (1.42 ± 0.03 at 1000 μg/mL; 1.38 ± 0.03 at 500 μg/mL and 1.16 ± 0.01 at 250 μg/mL) followed by hexane (0.85 ± 0.001), chloroform (0.22 ± 0.005) and acetone (0.12 ± 0.005) extracts at 1000 μg/mL (Fig. 2).

5.2.3. Osmotic fragility assay

The assay was performed to decipher the capability of different *W. fruticosa* leaf extracts in conferring resistance to the hemolysis of the RBC obtained from SCD patients. The result of the assay revealed that there was decrease in percent hemolysis in the RBC from SCD patient in the presence of *W. fruticosa* extracts. The decreasing percent of hemolysis was displayed in the presence of hexane, acetone and methanol extracts at varying concentration of NaCl solution. Complete osmotic fragility is usually observed at 0.4–0.3% NaCl concentration. The percent hemolysis observed at these NaCl percentages in the methanol and hexane extracts were 42.7 ± 0.6, 53.7 ± 0.6 and 41.2 ± 0.7, 48.2 ± 0.07 respectively. The percent hemolysis observed in all other tested extracts is appended in Fig. 3.

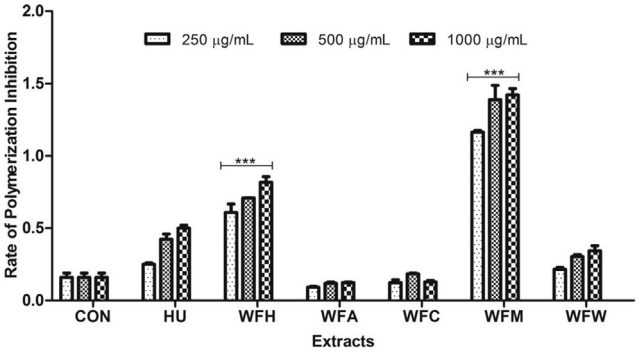

Fig. 2 Polymerization Inhibition Assay. *W. fruticosa* leaf extracts (250, 500 and 1000 μg/mL) were evaluated for their polymerization inhibition potential. Each value is represented as mean ± SEM of three replicate experiments. (*** represent $P < 0.05$ signifying highly significant results compared to standard). WFH (Hexane extract), WFA (Acetone extract), WFC (Chloroform extract), WFM (Methanol extract), WFW (Aqueous extract).

5.3. Evaluation of anti-inflammatory capacities of different *W. fruticosa* leaf extracts.

5.3.1. Membrane stabilization assay

Different extracts of *W. fruticosa* at different concentrations were tested for membrane stabilizing potential of RBC treated with hypo saline solution. Concentration dependent protection from hemolysis was observed in the methanol and aqueous extracts that was comparable to the standard drug indomethacin. The maximum protection at 1000 μg/mL in methanol (73.8%) and aqueous extracts (68.8%) was comparable to

Table 2 Reverse sickling analysis of *W. fruticosa* leaf extracts.

Concentration(μg/mL)	Percent of reverse sickling					
	HU	WFH	WFA	WFC	WFM	WFW
250	44.5 ± 1.5	34.5 ± 0.5	32 ± 1.0	30.5 ± 0.5	*59.7 ± 0.7****	43.5 ± 0.5
500	48 ± 1.0	38.2 ± 0.2	36.5 ± 0.5	33.4 ± 0.4	*63.5 ± 0.5****	48.5 ± 0.8
1000	49.5 ± 0.5	42.7 ± 0.7	39.9 ± 0.9	37.5 ± 0.5	*66 ± 1****	47.5 ± 0.5

Each value is represented as mean ± SEM of three replicate experiments. (*** represent $P < 0.05$ signifying highly significant results compared to standard (HU)). WFH (Hexane extract), WFA (Acetone extract), WFC (Chloroform extract), WFM (Methanol extract), WFW (Aqueous extract).

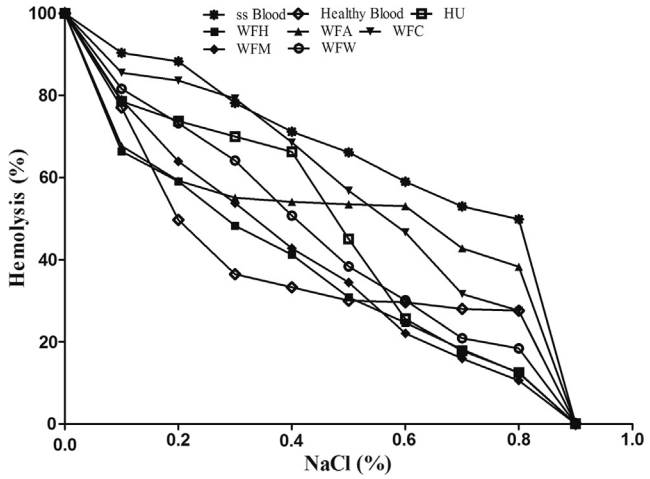

Fig. 3 Osmotic Fragility Assay. Different *W. fruticosa* extracts were evaluated for their cytotoxic potential in terms of hemolysis (%). All the values have been expressed as mean value obtained in three replicate experiments. Each value is represented as mean ± SEM of three replicate experiments. (*** represent $P < 0.05$ signifying highly significant results compared to standard). WFH (Hexane extract), WFA (Acetone extract), WFC (Chloroform extract), WFM (Methanol extract), WFW (Aqueous extract).

the protection (77.4%) provided by the standard drug indomethacin (Fig. 4).

5.3.2. Lipoxygenase inhibition assay

Lipoxygenase inhibition potential of *W. fruticosa* leaf extracts was displayed by acetone and methanol extracts. Concentration dependent inhibition activity was observed in both these extracts that was comparable to the standard (15- Lipoxygenase standard) provided in the kit (Fig. 5). Hexane, chloroform and aqueous extracts were not able to inhibit the lipoxygenase enzyme.

5.3.3. Xanthine oxidase inhibition

Concentration dependent XO inhibition activity was primarily observed in the hexane (57.0 ± 0.5%) and chloroform (56.3 ± 0.6%) extracts while the inhibition obtained in methanol (46.3 ± 0.8%), aqueous extract (35 ± 0.5%) and acetone (5.8 ± 0.2) were lower. The inhibition observed in the standard drug allopurinol was highest compared to all the extracts at all concentrations (Fig. 6).

5.4. Evaluation of antioxidant capacities different *W. fruticosa* leaf extracts

5.4.1. DPPH assay

DPPH basically measures the free radical scavenging capacity of antioxidants and is a commonly used method. The IC_{50} values of different extracts have been appended in Table 3. The methanol extract displayed significant IC_{50} values (8.1 ± 1.5 μg/mL) as compared to the standard gallic acid (39.0 ± 0.6 μg/mL).

5.4.2. ABTS assay

The IC_{50} values for different extracts were evaluated (Table 3). The results conferred that the acetone extract was most potent scavenger of the free radical with IC_{50} values of 215.8 ± 5.7 μg/mL. Hexane, chloroform, methanol and aqueous extract of *W. fruticosa* displayed lower scavenging activities as compared to those of standard ascorbic acid (IC_{50}: 284.7 ± 0.9 μg/mL).

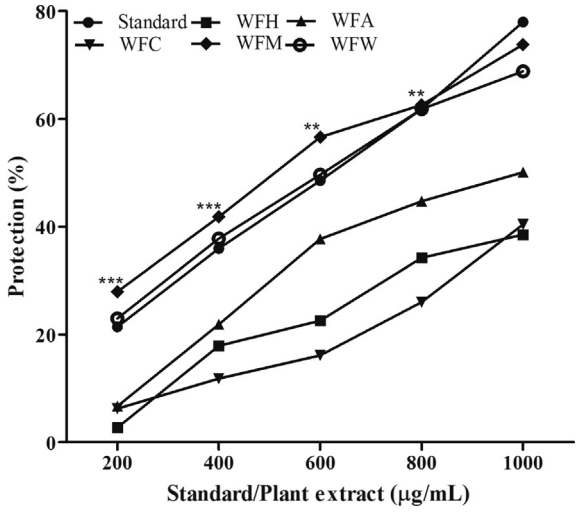

Fig. 4 Membrane stabilization potential of *W. fruticosa* extracts. Different *W. fruticosa* extracts were analyzed at 200, 400, 600, 800, 1000 μg/mL for analysis of RBC protection from hemolysis. The results are presented in terms of percent protection in hemolysis. Each value is represented as mean ± SEM of three replicate experiments. (*** represent $P < 0.05$ signifying highly significant results compared to standard). WFH (Hexane extract), WFA (Acetone extract), WFC (Chloroform extract), WFM (Methanol extract), WFW (Aqueous extract).

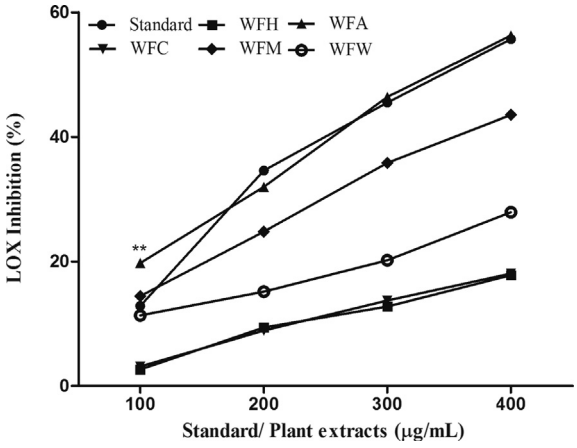

Fig. 5 Lipoxygenase Inhibition Assay. Different *W. fruticosa* extract and standard drug were analyzed for inhibition of the lipoxygenase enzyme analyzed at 100, 200, 300, 400 μg/mL. Each value represents the mean value of the three replicate experiments. Each value is represented as mean ± SEM of three replicate experiments. (*** represent $P < 0.05$ signifying highly significant results compared to standard). WFH (Hexane extract), WFA (Acetone extract), WFC (Chloroform extract), WFM (Methanol extract), WFW (Aqueous extract).

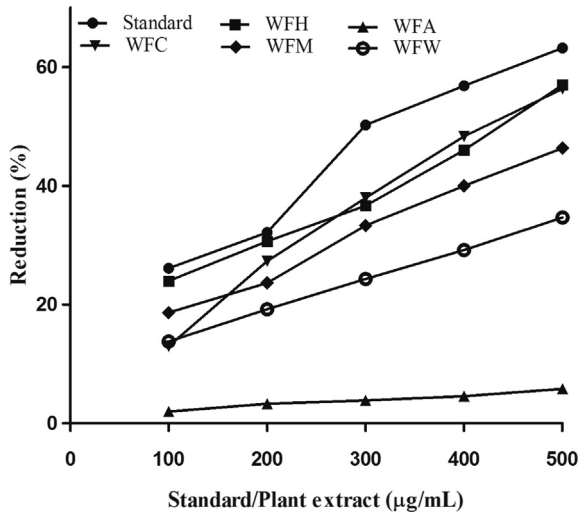

Fig. 6 Xanthine Oxidase Inhibition Assay. Different *W. fruticosa* extract and standard drug were analyzed for inhibition of the Xanthine oxidase at 100, 200, 300, 400, 500 µg/mL. Each value represents the mean value of the three replicate experiments. Each value is represented as mean ± SEM of three replicate experiments. (*** represent $P < 0.05$ signifying highly significant results compared to standard). WFH (Hexane extract), WFA (Acetone extract), WFC (Chloroform extract), WFM (Methanol extract), WFW (Aqueous extract).

5.4.3. FRAP assay

Ferrous Reducing antioxidant power of plant extracts of *W. fruticosa* was measured and a concentration dependent response was observed in hexane, aqueous, methanol and standard groups. FRAP value or reducing (Fe (III) to Fe (II)) capacity of Hexane extract was highest (1.5 ± 0.5 mM (Fe (II)/g dry weight) followed by methanol (1.45 ± 0.1 mM (Fe (II)/g dry weight) and aqueous (1.45 ± 0.05 mM (Fe(II)/g dry weight) extracts at the highest tested concentration (500 µg/mL). The FRAP values were comparable to those observed in the standard ascorbic acid (0.93 ± 0.01 mM (Fe (II)/g dry weight). Acetone and chloroform extracts displayed least FRAP values (Fig. 7).

5.4.4. TPC

The results of the assay revealed that methanol extract of *W. fruticosa* displayed highest phenolic content (113.7 ± 0.7) followed by acetone (68.1 ± 5.6), chloroform (22.4 ± 0.7), hexane (15.9 ± 0.7) and aqueous (7.2 ± 0.7) extracts (Table 3). The total phenolic content has been reported in terms of mg gallic acid equivalents/g dry material.

5.4.5. TFC

As a basis quantitative determination, Total flavonoid content in the *W. fruticosa* extracts was determined using aluminium chloride colorimetric method. The results have been expressed in quercetin equivalents (QE) per gram dry extract weight (Table 3). The flavonoid content in methanol extract was found to be highest 156.9 mg QE/g, while lowest flavonoid content was present in the hexane extract (0.45 ± 0.1) representing a great variation among the different extracts (Table 3).

5.4.6. TTC

The acetone extract of *W. fruticosa* had maximum total tannin content (365 ± 2.4), followed by methanol (338.1 ± 7.0), aqueous (101.2 ± 1.2), chloroform (15.8 ± 4.3) and hexane extracts (12.8 ± 9.0) (Table 3) The total tannin content has been reported in terms tannic acid equivalents/g dry weight.

5.5. Chemical characterization of *W. fruticosa* methanol leaf extract.

5.5.1. Chemical profiling by LC-MS

Methanol extract of *W. fruticosa* was most active in terms of anti-sickling, anti-oxidant and anti-inflammatory activities. It was therefore selected for LC-MS analysis for identifying bioactive compounds that might be responsible for observed activities. The LC-MS analysis is based on the comparative analysis of the mass of the currently known compounds obtained from the literature, databases or libraries. The results revealed a total of eighty two compounds from those we were able to identify thirty six compounds (Table 4, Fig. 8). LC-MS result demonstrated the presence of many compounds like kempferol, β sitosterol and quercetin that are previously reported to have anti-sickling properties. Numerous quercetin derivatives are present in the fraction such as quercetin 3-O-(6″-galloyl)-D galactopyranoside, quercetin 3-O-(6″-galloyl)-D galactopyranoside, quercetin, quercetin 3-O-D-galactoside, quercetin 3-L-arabinoside, quercetin 3-rhamnoside, quercetin 3-L-arabinoside, quercetin 3-rhamnoside. Out of the total 82 compounds obtained forty seven compounds are unknown

Table 3 Anti-oxidant analysis of *W. fruticosa* leaf extracts.

Plant Extract	TPC (mg Gallic acid Equivalents/g dry material)	TFC (mg Quercetin Equivalents/g dry material)	TTC (mg Tannic acid Equivalents/g dry material)	IC_{50} DPPH (µg/mL)	IC_{50} ABTS (µg/mL)
Standard	–	–	–	39.0 ± 0.6	284.7 ± 0.9
WFH	15.9 ± 0.7	0.45 ± 0.1	12.8 ± 9.0	260.9 ± 7.9	437.9 ± 3.9
WFA	68.1 ± 5.6	56.9 ± 1.2	365 ± 2.4	–	*215.8 ± 5.7**
WFC	22.4 ± 0.7	0.87 ± 0.2	15.8 ± 4.3	39.3 ± 0.3	337.0 ± 23.9
WFM	113.7 ± 0.7	156.9 ± 2.0	338.1 ± 7.0	*8.1 ± 1.5****	367.0 ± 0.0
WFW	7.2 ± 0.7	0.54 ± 0.04	101.2 ± 1.2	36.5 ± 0.6	417.0 ± 1.6

Each value is represented as mean ± SEM of three replicate experiments. (*** represent $P < 0.05$ signifying highly significant results compared to standard). WFH (Hexane extract), WFA (Acetone extract), WFC (Chloroform extract), WFM (Methanol extract), WFW (Aqueous extract).

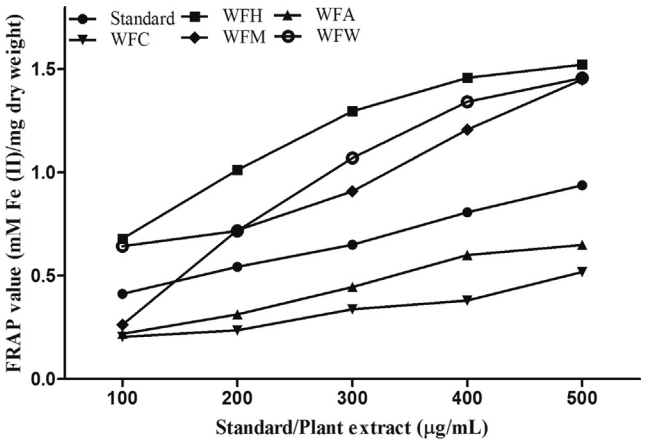

Fig. 7 FRAP Assay. FRAP value (mM (Fe(II)/g dry weight) of different *W. fruticosa* extracts were analyzed at 100, 200, 300, 400, 500 µg/mL. Each value is represented as mean ± SEM of three replicate experiments. (*** represent $P < 0.05$ signifying highly significant results compared to standard). WFH (Hexane extract), WFA (Acetone extract), WFC (Chloroform extract), WFM (Methanol extract), WFW (Aqueous extract).

that may play crucial role in anti-sickling, anti-oxidant and anti-inflammatory activities.

6. Discussion

Traditional use of medicinal plants for the treatment of SCD has led to productive results proving helpful in crisis reduction and also *in vitro* reverse sickling. Jobelyn (*Sorghum bicolor*), Ciklavit (*Cajanus cajan*), Dioscovite, *Carica papaya* leaf extract and Niprisan (*Piper guineense*, *Pterocarpus osun, Eugenia caryophyllum,* and *S. bicolor*) are such herbal products that are widely used for the treatment of SCD (Imaga N et al., 2011; Imaga N., 2013). These herbal drugs serve as agents that alter membrane stability, reduce hemolysis and osmotic fragility, inhibit HbS polymerization, or reverse the RBC sickling (Nurain et al., 2017). There has been an increased interest in determination of potent naturally occurring anti-sickling agents which could overcome the debilitating effects of available drugs and the high costs associated with the SCD management and treatment (Shah et al., 2020). In the current study *W. fruticosa*, a traditionally used medicinal plant by the tribal communities of Amarkantak region was evaluated for its anti-sickling, anti-inflammatory and anti-oxidant potential. A wide range of chemical compounds including alkaloids, tannins, saponin, flavonoids, glycosides, steroids and polyphenols were present in different extracts that were in line with previous reports (Birajdar et al., 2014). The numerous biological and therapeutic properties associated with these phytoconstituents may be responsible for the observed activities in *W. fruticosa* leaf extracts. Naturally occurring compounds having antisickling properties are rich in aromatic amino acids, phenolic compounds, flavonoids and antioxidant nutrients and are known to display anti-sickling properties either by inducing HbF production, by increasing the delay time required for HbS polymerization or by affecting the erythrocyte membrane leading to low amounts of intracellular Hb concentration and thereby inhibiting polymerization (Imaga and Taiwo., 2017). Similarly properties of plant substances that are rich in antioxidants and other nutritional components and their possible effects in the prevention and control of various diseases can be evaluated (Mollica A et al., 2017). Diets rich in anti-oxidants, amino acids, vitamins (A, C, and E), selenium or zinc can provide relief to the SCD individuals and prevent crisis (Noguchi CT and Schechter AN. 1978).

The methanol extract of *W. fruticosa* leaves displayed most prominent activities in anti-sickling assays. HU known to increase increased expression of fetal hemoglobin (HbF), RBC deformability, decrease the expression of adhesion molecules on reticulocytes and reduce the translocation of phosphatidylserine from inner to outer leaflet of RBC membrane lipid bilayer was used as was used as a standard in all the anti-sickling assays. The methanol extract displayed significant ($p < 0.05$) reverse sickling properties compared to those of HU. The observed activity in plant extracts might be due to the diversity of plant extracts present in them which must be further isolated, purified and tested for their potential in reverse sickling. Compared to the HU or control, the methanol extract displayed significant polymerization inhibition. Due to Hb polymerization the permeability of the RBC membranes is also altered and thus the cation leakage. Increased Ca_2^+ levels are known to damage the normal cell shape and plasticity, increased K + leakage and inhibiting Na^+, K^+ pump. Natural products from plants due to their diverse nature can interfere with the Hb polymerization, alter the permeability of the RBC or may regulate the elevated levels of Ca_2^+ and might provide us with novel treatment strategies for the control or management of SCD. Some phytoconstituents such as terpenoids, polyphenols, phenylalanine and hydroxybenzoic acid components possess anti-sickling properties (Ogoda et al., 2002; Biapa et al., 2019). Due to the occurrence of inflammation mediated pathophysiology in SCD patients, current therapeutic approaches targeting molecules of inflammatory processes seems to be a viable approach. The anti-inflammatory drugs might work by ameliorating a broad range of inflammatory mechanisms including reduction of leukocyte adhesion to the blood vessel wall suppressing the vaso-occlusive process or by neutralizing important players in the SCD inflammatory scenario or specific inflammatory cell types (Ghosh et al., 2013; Belcher et al., 2014). The membrane stabilization assay displayed that the methanol and aqueous extracts of *W. fruticosa* prevented hemolysis of RBC that were comparable to the standard group. Lysosomal enzymes released due to defects in lysosomes leads to abnormal autophagy, activation of inflammation, and reduced infection control (Chowdhury et al., 2014; Simonaro 2016). The methanolic and aqueous extracts that were capable of RBC membrane stabilization will also stabilize lysosome membrane due to its structural similarity to RBC membrane. LOX metabolizes arachidonic acid leading to the production of leukotrienes that are known to be the mediators of inflammation (Owusu-Ansah et al., 2016). Inhibitors of LOX can reduce the production of leukotrines and thus inflammation. Several studies have demonstrated the inhibitory activities from methanol and aqueous extracts of *W. fruticosa* flowers (Kumaraswamy and Satish 2008). Molecules such as pelargonidin, quercetin and myricetin have been identified from *W. fruticosa* flowers that might be responsible for observed anti-inflammatory activity (Gabor and Razga 1991; El-Seedi et al., 2003). During vasoocclusive crisis higher levels of XO are observed that have

Table 4 LC-MS analysis of methanolic leaf extract of *W. fruticosa*.

Peak No.	RT (Min)	Molecular weight (g/mol)	Name of the Compound	LC-MS (Obtained mass, *m/z*)	Class of Compounds	Molecular Formula
Positive mode (ES +)						
1	1.11		Unknown	381.1		
2	4.43	168	1-Cyclohexene-1-methanol, alpha.,2,6,6-tetramethyl-	169.0 (M + 1)	Monoterpenoid	$C_{11}H_{20}O$
3	5.32	228	Tetradecanoic acid	229.1 (M + 1)	Saturated fatty acid	$C_{14}H_{28}O_2$
4	5.46		Unknown	888.5 (M + 1)		
5	6.79	616.5	Quercetin 3-O-(6″-galloyl)-D – galactopyranoside	617.2 (M + 1)	Flavonoid	
6	7.52, 11.12	302.19	Ellagic acid	303.1 (M + 1)	Phenolic Compound	$C_{14}H_6O_8$
7	8.03	434.3	Quercetin 3-L-arabinoside	435.1 (M + 1)	Flavonoid	$C_{20}H_{18}O_{11}$
8	8.43	448.4	Quercetin 3-rhamnoside	449.2 (M + 1)	Flavonoid	$C_{21}H_{20}O_{11}$
9	9.40		Unknown	287.1 (M + 1)		
10	13.92	180	Dihydroactinidiolide	181.1 (M + 1)	Terpenes	$C_{11}H_{16}O_2$
11	14.30	220	Caryophyllene Oxide/Caryophyllene Epoxide	221.1 (M + 1)	Terpenes	$C_{15}H_{24}O$
12	14.67	256	Hexadecanoic acid	259.1 (M + 3)	Saturated fatty acid	$C_{16}H_{32}O_2$
13	15.31	430.6	Hecogenin	453.3 (M + Na)	Steroid	$C_{27}H_{42}O_4$
14	15.66	272	(E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene/β-Springene	274.3 (M + 2)	Aliphatic and hydrocarbonic diterpene	$C_{20}H_{32}$
15	16.14	416.4	Chrysophanol-8-O-D-Glucopyranoside	417.2 (M + 1)	Bicyclic flavonoids	$C_{21}H_{20}O_9$
16	16.31	340	Eicosanoic acid, ethyl ester/Arachidic acid	341.1 (M + 1)	Saturated fatty acid	$C_{22}H_{44}O_2$
17	16.45		Unknown	855.4		
18	16.85	310	Ethyl Oleate 9-Octadecenoic acid (Z)-	354.4 (M + 2Na-1)	Fatty acid	$C_{22}H_{43}NO_3$
19	17.30	456.7	Betulinic acid	455.3 (M + 1)	Triterpenoid	$C_{30}H_{48}O_3$
20	18.79	662.8	Myricetin 3-O- L-arabinopyranoside	664.5 (M + 1)	Flavonol glycoside	$C_{20}H_{18}O_{12}$
21	19.08		Unknown	532.4 (M + 1)		
22	19.52		Unknown	352.4 (M + 1)		
23	19.67		Unknown	352.3 (M + 1)		
24	19.81		Unknown	689.5 (M + 1)		
25	19.96, 22.05	611.5	Cyanidin 3,5-diglucoside	644.6 (M + 1 + MeOH)	Anthocyanin	$C_{27}H_{31}O_{16}^{+}$
26	20.08		Unknown	600.5 (M + 1)		
27	20.25		Unknown	556.4 (M + 1)		
28	20.39	448.4	Kaempferol 3-O-glucoside	512.4 (M + Na + CAN)	Phenolic compound	$C_{21}H_{20}O_{11}$
29	20.58		Unknown	722.5 (M + 1)		
30	20.74	634.5	Gemin D	678.5 (M + 2Na-1)	Hydrolyzable tannins	$C_{27}H_{22}O_{18}$
31	21.03		Unknown	590.5 (M + 1)		
32	21.88		Unknown	353.3 (M + 1)		
33	23.92		Unknown	353.4 (M + 1)		
34	24.31	346	6,9,12,15-Docosatetraenoic acid, methyl ester	397.2 (M + 1 + MeOH)	Polyunsaturated fatty acid (ω-6 fatty acid)	$C_{23}H_{38}O_2$
35	24.77		Unknown	600.5 (M + 1)		
36	24.95		Unknown	353.3 (M + 1)		
37	25.51	292	Benzenepropanoic acid / Hydrocinnamic acid/ 3,5-bis(1,1-dimethylethyl)-4-hydroxy-	325.4 (M + 1 + MeOH)	Phenylpropanoids	$C_9H_{10}O_2$
38	26.05		Unknown	353.4 (M + 1)		
39	26.26		Unknown	568.5 (M + 1)		
40	27.94		Unknown	593.3 (M + 1)		
41	28.41		Unknown	688.5 (M + 1)		
Negative mode (ES-)						
1	1.14		Unknown	341.1 (M−1)		
2	1.58	314.24	Norbergenin	313.1 (M−1)	C-glycoside	$C_{13}H_{14}O_9$
3	2.86	784.5	Oenothein-C	783.2 (M−1)	Ellagitannin Oligomer	$C_{34}H_{24}O_{22}$

Table 4 (*continued*)

Peak No.	RT (Min)	Molecular weight (g/mol)	Name of the Compound	LC-MS (Obtained mass, *m*/*z*)	Class of Compounds	Molecular Formula
4	3.96		Unknown	289.1 (M−1)		
5	4.44		Unknown	329.1 (M−1)		
6	5.14	434.3	Quercetin 3-O-D-xylopyranoside	432.3 (M−2)	Flavonoid	$C_{20}H_{18}O_{11}$
7	5.29		Unknown	431.3 (M−1)		
8	5.47		Unknown	859.2 (M−1)		
9	6.14	414.7	β-sitosterol	412.1 (M−2)	Phytosterol	$C_{29}H_{50}O$
10	6.53		Unknown	468.2 (M−1)		
11	6.80, 7.01	616.5	Quercetin 3-O-(6″-galloyl)-D-galactopyranoside	615.1 (M−1)	Flavonoid	$C_{28}H_{24}O_{16}$
12	7.24	302.23	Quercetin	301.0 (M−1)	Flavonoid	$C_{15}H_{10}O_7$
13	7.53	464.4	Quercetin 3-O–D-galactoside	463.2 (M−1)	Flavonoid	
14	8.04	434.3	Quercetin 3–L-arabinoside	433.1 (M−1)	Flavonoid	$C_{20}H_{18}O_{11}$
15	8.42	448.4	Quercetin 3-rhamnoside	447.1 (M−1)	Flavonoid	$C_{21}H_{20}O_{11}$
16	9.41		Unknown	431.2 (M−1)		
17	9.83		Unknown	191.0 (M−1)		
18	10.30		Unknown	477.3 (M−1)		
19	11.11	302.23	Quercetin	301.0 (M−1)	Flavonoid	$C_{15}H_{10}O_7$
20	12.69		Unknown	327.3 (M−1)		
21	13.33		Unknown	329.3 (M−1)		
22	14.51		Unknown	353.1 (M−1)		
23	14.66	260	2H-1-Benzopyran-2-one	259 (M−1)	Phenolic compound	$C_9H_6O_2$
24	15.32		Unknown	487.4 (M−1)		
25	15.57		Unknown	377.1 (M−1)		
26	16.15, 21.23	416.4	Chrysophanol-8-O—D Glucopyranoside	415.2 (M−1)	Bicyclic flavonoids	$C_{21}H_{20}O_9$
27	16.32		Unknown	337.1 (M−1)		
28	17.31	374	10,12-Pentacosadiynoic acid	372.2 (M−2)	Amphiphilic diacetylene	$C_{25}H_{42}O_2$
29	17.87		Unknown	385.2 (M−1)		
30	18.02		Unknown	471.4 (M−1)		
31	19.97		Unknown	399.3 (M−1)		
32	21.48		Unknown	369.2 (M−1)		
33	21.60		Unknown	311.3 (M−1)		
34	22.27		Unknown	369.2 (M−1)		
35	23.86		Unknown	455.4		
36	25.21		Unknown	567.4		
37	25.63		Unknown	821.4 (M−1)		
38	26.99		Unknown	437.3 (M−1)		
39	27.95		Unknown	591.3 (M−1)		
40	30.75	256	Hexadecanoic acid	255.3 (M−1)	Saturated fatty acid	$C_{16}H_{32}O_2$
41	33.30		Unknown	569.4 (M−1)		

been linked to increased levels of ROS, inflammation, tissue injury and impaired vascular relaxation in SCD (Odebiyi and Fasola, 2020). Allopurinol, an XO inhibitor was reported to reduce the oxidative stress in sickle cell erythrocytes in a murine model of SCD via a direct scavenging mechanism (Pacher et al., 2006). A recent study suggested that inhibition of XO decreased the hemolysis in bone marrow transplanted chimeric SCD mice (Schmidt et al., 2020). A large number of medicinal plants have been validated to possess XO inhibitors. We observed moderate XO inhibitory activities in the hexane and chloroform extract of *W. fruticosa* leaf extracts that were comparable to that of the standard drug Allopurinol. Previous reports on anti-inflammatory effects of *W. fruticosa* also support our findings (Hiralal et al., 2014; Raj et al., 2020). Several studies have reported antioxidant molecules as potent inhibitors of HbS polymerization and increase in the oxidant status of sickled erythrocytes (Stuart et al., 1994; Imaga et al., 2011; Nwaoguikpe and Braide 2012). The higher the levels of the antioxidants in the plants extracts, the higher is the possibility of them displaying anti sickling properties that might be due to reduction in oxidative stress, enhanced membrane stabilizing capabilities or by donation of the free electron to the iron molecule of Hb (Devi et al., 2008). The use of *W. fruticosa* by the traditional healers is justified due to the evidence of high levels of antioxidant activities observed in the methanol and hexane extracts that were supported by the high levels of total phenolics, flavonoids and tannin content observed in our current study. Similar results were observed in a recent study where the methanolic extracts displayed significant antioxidant activities that correlated with

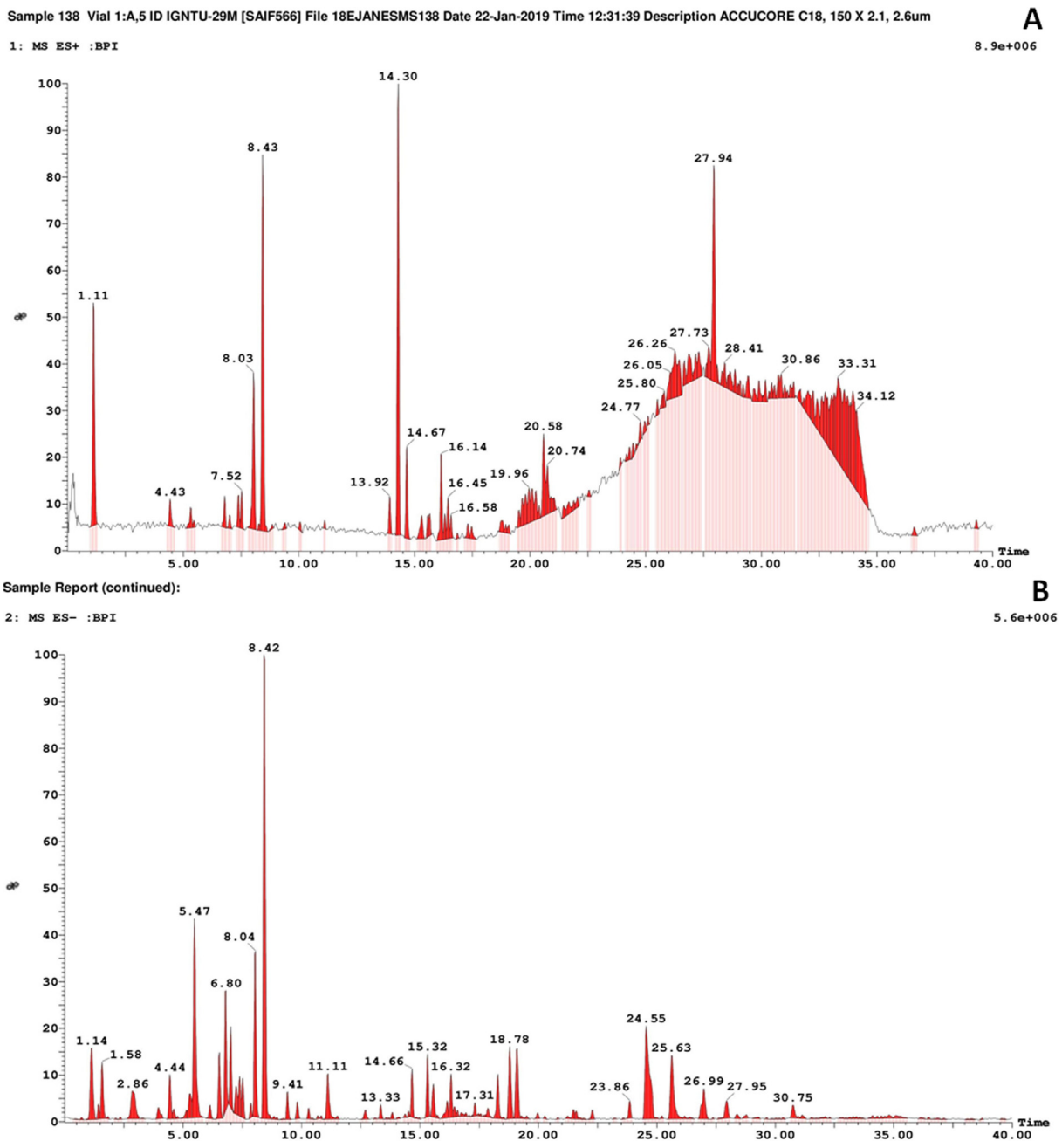

Fig. 8 LC-MS chromatogram of *W. fruticosa* methanolic leaf extract**.** Chromatogram in positive ion mode **(A)** and Chromatogram in negative ion mode **(B)**.

high concentrations of total flavonoid and phenolic content (Neupane and Lamichhane 2020). In earlier studies, antioxidant capacity has been reported from *W. fruticosa* leaves, flowers and bark (Pawar et al., 2011; Finose and Devaki 2011; Chaturvedi et al., 2012). The antioxidant capacity depends on various mechanisms such as inhibition of generation and scavenging capacity against ROS/RNS, reducing capacity, metal chelating capacity, activity as anti-oxidative enzyme and inhibition of oxidative enzymes (Magalhães LM et al., 2008) and due to this reason we might have observed differential results in our antioxidants assays. FRAP assay is based on the ability of the antioxidant to reduce Fe(3 +) to Fe(2 +) through the donation of an electron similarly DPPH or ABTS measure the capability to donate an electron to an DPPH or ABTS free radical. All the reactions are measured at different wavelengths and some of the phytoconstituents might interfere in some specific assay. The oxidative stress experienced by the SCD patients related to severe complications due to the increased hemolysis, RBC deformability can be relieved by the use of extracts of *W. fruticosa* that can contribute to the effective management of SCD. For identification of potent metabolites in the most active methanol fraction of

W. fruticosa leaf extract LC-MS studies were undertaken where 41 compounds were identified in positive ion mode and 41 in negative ion mode respectively. Some of the identified bioactive compounds have been previously reported for many biological activities such as anti-inflammatory, antimicrobial, anti-oxidant as well as anti-sickling properties. Quercetin is an antioxidant flavonoid with a potential to protect against Hb oxidation, the binding of Hb to membrane and lipid peroxidation thereby reducing oxidative damage and inflammation in SCD patients (Cesquini M et al., 2003). Quercetin is also reported to inhibit the generation of O_2^- by XO inhibition (Zhang et al., 2018). Quercetin was also found to reduce hydroxyurea induced cytotoxicity in immortalized mouse aortic endothelial cells (Kiser ZM et al., 2017). Oenothein are reported to be antioxidant and anti-inflammatory in nature (Yoshida T et al., 2018). Kaempferol is known to possess anti sickling and antioxidant properties but was found to be toxic to RBC in hemolysis assay. Triterpenoids such betulinic acid, maslinic acid, oleanolic acid and their esters isolated from *Melaleuca bracteata* and *Syzygium aromaticum* have been reported to have anti-sickling and antioxidant properties (Folashade and Omoregie 2013). Betulinic acid was also reported to be active in theoretical reactivity studies while β-sitosterol was found to be unreactive to the HbS (Muya, JT et al., 2019). Ellagic acid derivatives isolated from *Combretum racemosum* have also displayed significant anti-sickling activities (Famojuro TI et al., 2021). The phytocannabinoids and vanilloids in *Eugenia caryophyllata and Piper guineense* may account for the useful effects of Niprisan in sickle cell crisis. β-Caryophyllene, an cannabinoididentified in our active extract is also found in *P. Guineense* and is known to selectively bind to CB2 receptors involved in SCD pain crises and thus can provide relief to SCD patients (Ameh SJ et al., 2012). Hexadecanoic acid and Octadecanoic acid has been reported from the extracts of *Telfairia occidentalis*, *Anacardium occidentale* and *Terminalia catappa* that stabilized sickle erythrocyte membrane against osmotic stress (Cyril-Olutayo MC et al., 2018; Chikezie, PC et al., 2020). Methylated esters such as methyl tetradecanoate, hexadecanoic acid, methyl ester, pentadecanoic acid, 14-methyl-methyl ester, 9, 12-octadecadienoic acid, (Z, Z)-methyl ester, 11-octadecenoic acid, methyl ester, and 9-octadecenoic acid, methyl ester (E)-from the petroleum ether extract of *Anacardium occidentale* attenuated HbS aggregation and polymerization (Chikezie PC et al., 2020). LC-MS analysis confirmed the presence of several other bioactive compounds that have been previously validated for numerous biological activities. The possible affinity of these molecules against HbS or the inhibitory action against XO or LOX can be inferred using multifunctional approaches such as molecular docking (Uysal A et al., 2019; Sadeer NB et al., 2019). The interaction of phytochemicals with the HbS molecule is the probable basis of reversal of HbS polymerization which is the main reason for observed pathophysiology in SCD patients. The use of phytochemicals that covalently bind and modify HbS molecules seems to be a viable approach for polymerization inhibition or reversal offering us novel therapeutic options for the treatment of SCD. The novel compounds present in the methanol extract are required to be identified and characterized for anti-sickling properties so that a novel anti sickling agent could be developed.

7. Conclusion

W. fruticosa is still used in several traditional medicinal systems and have been reported for anti-bacterial, anti-tumor, anti-inflammatory and many more activities. Pharmacological and biochemical investigation of leaves of *W. fruticosa* revealed the presence of almost all the major phytoconstituents including alkaloids, flavonoids, tannin, saponin, glycoside, steroid and phenolic in all tested extracts. Methanol extract is the most potent extract having significant reverse sickling, polymerization inhibition, osmotic fragility and anti-inflammatory, anti-oxidant activities. The known and unknown compounds identified by LC-MS analysis need to be purified and further characterized for anti-sickling potential so that new therapeutic agents could be developed that could help in control or management of SCD. The results of the current study validate the traditional use of *W. fruticosa* by tribal communities.

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Ethics approval and consent to participate

Human blood samples were obtained from the District hospital, Anuppur, MP after getting prior approval from the IGNTU-Institutional Human Ethics Committee (Ref: IEC No. Project Proposal Id: 003/IGNTU-IEC/2019-01) dated 16-02-19. The blood samples have been collected from the patient after the proper concern through consent form.

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Availability of data

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2021.103539.

References

Strader, M.B., Jana, S., Meng, F., et al, 2020. Post-translational modification as a response to cellular stress induced by hemoglobin oxidation in sickle cell disease. Sci Rep. https://doi.org/10.1038/s41598-020-71096-6.

Yuan, H., Ma, Q., Ye, L., et al, 2016. The Traditional Medicine and Modern Medicine from Natural Products. Molecules. https://doi.org/10.3390/molecules21050559.

Adejumo, O.E., Kolap, A.L., Roleola, O.P., et al, 2010. In vitro antisickling activities and phytochemical evaluation of Plumbago zeylanica and Uvaria chamae. Afr. J. Biotechnol. 9 (53), 9032–9036.

Hockham, C., Bhatt, S., Colah, R., et al, 2018. The spatial epidemiology of sickle-cell anaemia in India. Sci Rep. https://doi.org/10.1038/s41598-018-36077-w.

Ogwutum FE, Augustine AU, Wegwu MO, *et al*. In vitro Investigation of the Anti-sickling and Erythrocyte Membrane Stabilizing Potentials of Elaesis guineensis Jacq Flower. Intr J Bioch Biophy. (2018). https://doi: 10.13189/ijbb.2018.060102.

Shah, F., Dwivedi, M., Parikh, C.V., et al, 2020. Promising anti-sickling and fetal hemoglobin inducing effects of *Boerhavia diffusa* root extract on sickle cell erythrocytes. J. Herb. Med. https://doi.org/10.1016/j.hermed.2020.100398.

Birajdar VV, Mhase AG, Gurav AM, *et al*. Preliminary pharmacognostic and phytochemical standardization of Dhataki [*Woodfordia fruticosa* (L.) Kurz.] leaves. Ayu. (2014). https://doi:10.4103/0974-8520.153752.

Das PK, Goswami S, Chinniah A, *et al*. Woodfordia fruticosa: traditional uses and recent. Ethnopharmacol. (2007). https://doi: 10.1016/j.jep.2006.12.029.

Kumar, D., Sharma, M., Sorout, A., et al, 2016. *Woodfordia fruticosa* Kurz.: A Review on its Botany, Chemistry and Biological activities. J. Pharmacogn Phytochem. 5, 293–298.

Katiyar, CK, Duggal RK, Venkata B, *et al*. Herbal composition and method of manufacturing such composition for the management of gynecological disorders. 2002. US Patent 6,455,077 B2.

Grover, N., Patni, V., 2013. Phytochemical characterization using various solvent extracts and GC-MS analysis of methanolic extract of *Woodfordia fruticosa* (l.) Kurz. Leaves. Int J Pharm Pharm Sci. 5 (4), 291–295.

Khan, I.A., Singh, A., Mindala, D.P., et al, 2019. Preclinical development of gastro-protective botanical candidate from Woodfordia fruticosa (Linn.) Kurz: Chemical standardization, efficacy, pharmacokinetics and safety pharmacology. J Ethnopharmacol.

Huie, C.W., 2002. A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. Anal Bioanal Chem. https://doi.org/10.1007/s00216-002-1265-9.

Zygmunt, B., Namieśnik, J., 2003. Preparation of samples of plant material for chromatographic analysis. J Chromatography Sci. https://doi.org/10.1093/chromsci/41.3.109.

Harborne, J.B., 1998. Textbook of Phytochemical Methods. In: A Guide to Modern Techniques of Plant Analysis. 5th Edition. Chapman and Hall Ltd London, pp. 21–72.

Senguttuvan, J., Paulsamy, S., Karthika, K., 2014. Phytochemical analysis and evaluation of leaf and root parts of the medicinal herb, Hypochaeris radicata L. for in vitro antioxidant activities. Asian Pac. J Trop Biomed. https://doi.org/10.12980/APJTB.4.2014C1030.

Nurain, I.O., Bewaji, C.O., Johnson, J.S., et al, 2017. Potential of Three Ethnomedicinal Plants as Antisickling Agents. Mol Pharm. https://doi.org/10.1021/acs.molpharmaceut.6b00767.

Nwaoguikpe RN, Ujowundu CO, Okwu GN, *et al*. The Antisickling Potentials of Four Curcubits (T. Occidentalis, C. Maxima; C. Sativus and C.Lonatus). Sch. J. App. Med. Sci. 1(3) (2013). 191-98.

Pauline, N., Cabral, B.N., Anatole, P.C., et al, 2013. The in vitro antisickling and antioxidant effects of aqueous extracts Zanthoxylum heitzii on sickle cell disorder. BMC Complement Altern Med. https://doi.org/10.1186/1472-6882-13-162.

Bolajoko, A.A., Amos, M.M., Musibau, O.I., et al, 2015. In vitro Evaluation of Membrane Stabilizing Potential of Selected Bryophyte Species. EJMPlants. 6 (3), 181–190.

Wahyuningsih, S., Sukandar, E.Y., Sukrasno, 2016. In Vitro Xanthine Oxidase Inhibitor Activity of Ethanol Extract and Fraction Roselle Calyx (Hibiscus sabdariffa L.). Int. J. Pharm. Clin. Res. 8 (6), 619–622.

Chang, S.T., Wu, J.H., Wang, S.Y., et al, 2001. Antioxidant Activity of Extracts from Acacia confusa Bark and Heartwood. J. Agric. Food Chem. https://doi.org/10.1021/jf0100907.

Boly, R., Lamkami, T., Lompo, M., et al, 2016. DPPH Free Radical Scavenging Activity of Two Extracts from *Agelanthus dodoneifolius* (Loranthaceae) Leaves. IJTP. 8 (1), 29–34.

Gan, R.Y., Xu, X.R., Song, F.L., et al, 2010. Antioxidant activity and total phenolic content of medicinal plants associated with prevention and treatment of cardiovascular and cerebrovascular diseases. J. Med. Plant. Res. 4 (22), 2438–2444.

Al-Abd, N.M., Mohamed Nor, Z., Mansor, M., et al, 2015. Antioxidant, antibacterial activity, and phytochemical characterization of Melaleuca cajuputi extract. BMC Complement Altern Med. https://doi.org/10.1186/s12906-015-0914-y.

Re, R., Pellegrini, N., Proteggente, A., et al, 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. https://doi.org/10.1016/s0891 5849(98)00315-3.

Arabshahi-Delouee, S., Urooj, A., 2007. Antioxidant properties of various solvent extracts of mulberry (Morus indica L.) leaves. Food Chem. 102 (4), 1233–1240.

El Jemli, M., Kamal, R., Marmouzi, I., et al, 2016. Radical-Scavenging Activity and Ferric Reducing Ability of Juniperus thurifera (L.). J. oxycedrus (L.), J. phoenicea (L.) and Tetraclinis articulate (L.). Adv Pharmacol Sci. https://doi.org/10.1155/2016/6392656.

Hossain, M.A., Shah, M.D., Gnanaraj, C., et al, 2011. In vitro total phenolics, flavonoids contents and antioxidant activity of essential oil, various organic extracts from the leaves of tropical medicinal plant *Tetrastigma* from Sabah. Asian Pac. J Trop. Med., 717–721

Quettier, D.C., Gressier, B., Vasseur, J., et al, 2000. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum Moench*) hulls and flour. J. Ethnopharmacol. 72, 35–42.

Muthukumaran P, Saraswathy N, Aswitha V, *et al*. Assessment of Total Phenolic, Flavonoid, Tannin Content and Phytochemical Screening of Leaf and Flower Extracts from Peltophorum pterocarpum (DC.) Backer ex K.Heyne: a comparative study. Pharmacogn. J. (2016). doi: 10.5530pj.2016.2.7.

Imaga Ngozi, A.O., Shaire, A.E., Ogbeide, S., et al, 2011. In vitro biochemical investigations of the effects of *Carica papaya* and *Fagara zanthoxyloides* on antioxidant status and sickle erythrocytes. Afr J Biochem Res. 5 (8), 226–236.

Imaga, N.A., 2013. Phytomedicines and nutraceuticals: alternative therapeutics for sickle cell anemia. Sci World J. https://doi.org/10.1155/2013/269659.

Imaga Ngozi, A., Oluwole, T., 2017. Different Therapeutic Interventions and mechanisms of action of antisickling agents currently in use in sickle cell disease management. EMJ Hematol. 51, 113–117.

Mollica, A., Zengin, G., Locatelli, M., et al, 2017. An assessment of the nutraceutical potential of Juglans regia L. leaf powder in diabetic rats. Food Chem Toxicol. https://doi.org/10.1016/j.fct.2017.03.056.

Noguchi, C.T., Schechter, A.N., 1978. Inhibition of sickle haemoglobin gelation by amino acids and related compounds. Biochemistry. 17 (25), 5455–5459.

Odebiyi, Ha, Fasola, Fa, 2020. Xanthine Oxidase Activity in Patients with Sickle Cell Anaemia Presenting with Vaso-Occlusive Crises in University College Hospital. Ibadan. AJLM. https://doi.org/10.11648/j.ajlm.20200502.13.

Ogoda, J.O., Akubue, P.I., Okide, G.B., 2002. The kinetics of reversal of pre-sickled erythrocytes by the aqueous extract of Cajanus cajan seeds. Phyto research. https://doi.org/10.1002/ptr.1026.

Biapa, P.C.N., Yembeau, N.L., Chetcha, B., et al, 2019. The anti-sickling mechanism of Amphimas pterocarpoides (Cesalpiniaceae) against sickle cell Anemia. Acad. J. Med. Plants 7 (4), 117–124.

Ghosh, S., Adisa, O.A., Chappa, P., et al, 2013. Extracellular hemin crisis triggers acute chest syndrome in sickle mice. J Clin Invest. https://doi.org/10.1172/JCI64578.

Belcher, J.D., Chen, C., Nguyen, J., et al, 2014. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. Blood. https://doi.org/10.1182/blood-2013-04-495887.

Chowdhury, A., Azam, S., Jainul, M.A., et al, 2014. Antibacterial Activities and In Vitro Anti- Inflammatory (Membrane Stability) Properties of Methanolic Extracts of Gardenia coronaria Leaves. Int. J Microbiol. https://doi.org/10.1155/2014/410935.

Simonaro, C.M., 2016. Lysosomes, Lysosomal Storage Diseases, and Inflammation. JIEMS. https://doi.org/10.1177/2326409816650465.

Owusu-Ansah, A., Ihunnah, C.A., Walker, A.L., et al, 2016. Inflammatory targets of therapy in sickle cell disease. Transl Res. https://doi.org/10.1016/j.trsl.2015.07.001.

Kumaraswamy, M.V., Satish, S., 2008. Free radical scavenging activity and lipoxygenase inhibition of *Woodfordia fructicosa Kurz* and *Betula utilis* Wall. Afr J Biotechnol. 7 (12), 2013–2016.

Gabor, M., Razga, Z., 1991. Effect of benzopyrone derivatives on simultaneously induced croton oil ear oedema and carrageenin paw oedema in rats. Acta Physiologica Hungarica. 77, 197–207.

El-Seedi, H.R., Ringbom, T., Torssell, K., et al, 2003. Constituents of Hypericum laricifolium and their cyclooxygenase (COX) enzyme activities. Chem Pharm Bull (Tokyo). https://doi.org/10.1248/cpb.51.1439.

Pacher, P., Nivorozhkin, A., Szabó, C., 2006. Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol. Pharmacol Rev.

Schmidt, H.M., Wood, K.C., Lewis, S.E., et al, 2020. Xanthine Oxidase Drives Hemolysis and Vascular Malfunction in Sickle Cell Disease. Arterioscler Thromb Vasc Biol.

Hiralal, G.M., Bhusari, K.P., Duragkar, N.J., et al, 2014. Pharmacological evaluation for anti-asthmatic and anti-inflammatory potential of *Woodfordia fruticosa* flower extracts. Pharm Biol. https://doi.org/10.3109/13880209.2013.869232.

Raj, H., Gupta, A., Upmanyu, N., 2020. Anti-inflammatory Effect of *Woodfordia fructicosa* Leaves Ethanolic Extract on Adjuvant and Carragenan Treated Rats. Antiinflamm Antiallergy Agents Med Chem. https://doi.org/10.2174/1871523018666190222120127.

Stuart, J., Mojiminiyi, F.B., Stone, P.C., et al, 1994. Additive in vitro effects of anti-sickling drugs. Br J Haematol. https://doi.org/10.1111/j.1365-2141.1994.tb04836.x.

Nwaoguikpe, R.N., Braide, W., 2012. The antisickling effects of some micronutrients and antioxidant vitamins in sickle cell disease management. Med Medic Sc J. 3 (5), 334–340.

Devi, K.P., Suganthy, N., Kesika, P., et al, 2008. Bioprotective properties of seaweeds: in vitro evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. BMC Complement Altern Med. https://doi.org/10.1186/1472- 6882-8-38.

Neupane, P., Lamichhane J. Estimation of total phenolic content, total flavonoid content and antioxidant capacities of five medicinal plants from Nepal. Vegetos. (2020). doi.org/10.1007/s42535-020-00116-7.

Pawar, S., Chaudhari, P., Shah, V., et al, 2011. In-vitro antioxidant activity of *Woodfordia fructicosa* flowers. J. Pharm. Res. 4 (10), 3498–3500.

Finose, A., Devaki, K., 2011. Phytochemical and Chromatographic Studies in the Flowers of *Woodfordia fruticosa (L) kurz*. Asian J Plant Sci Res. 1 (3), 81–85.

Chaturvedi PA, Ghatak AA, Desai NS. Evaluation of radical scavenging potential and total phenol content in Woodfordia fruticosa from different altitudes. J. Plant Biochem. Biotechnol. (2012). doi.org/10.1007/s13562-011-0066-1.

Magalhães, L.M., Segundo, M.A., Reis, S., et al, 2008. Methodological aspects about in vitro evaluation of antioxidant properties. Anal Chim Acta. https://doi.org/10.1016/j.aca.2008.02.047.

Cesquini, M., Torsoni, M.A., Stoppa, G.R., et al, 2003. t-BOOH induced oxidative damage in sickle red blood cells and the role of flavonoids. Biomed. Pharmacother. 57, 124–129.

Zhang, C., Wang, R., Zhang, G., et al, 2018. Mechanistic insights into the inhibition of quercetin on xanthine oxidase. Int. J. Biol. Macromol. 112, 405–412. https://doi.org/10.1016/j.ijbiomac.2018.01.190.

Kiser, Z.M., McGee, M.D.M., Wright, R.J., et al, 2017. Quercetin reduces hydroxyurea induced cytotoxicity in immortalized mouse aortic endothelial cells. PeerJ. https://doi.org/10.7717/peerj.3376.

Yoshida, T., Yoshimura, M., Amakura, Y., 2018. Chemical and Biological Significance of Oenothein B and Related Ellagitannin Oligomers with Macrocyclic Structure. Molecules. https://doi.org/10.3390/molecules23030552.

Folashade, K.O., Omoregie, E.H., 2013. Chemical constituents and biological activity of medicinal plants used for the management of sickle cell disease - A review. JMPR. https://doi.org/10.5897/JMPR2013.5333x.

Muya, J.T., Mwanangombo, D.T., Tsalu, P.V., et al, 2019. Conceptual DFT study of the chemical reactivity of four natural products with anti-sickling activity. SN Appl. Sci. https://doi.org/10.1007/s42452-019-1438-8.

Famojuro, T.I., Adeyemi, A.A., Ajayi, T.O., et al, 2021. Anti-sickling activities of two isolated compounds from the root of *Combretum racemosum P. beauv*. (Combretaceae). J Ethnopharmacol. https://doi.org/10.1016/j.jep.2021.113992.

Ameh, S.J., Tarfa, F.D., Ebeshi, B.U., 2012. Traditional Herbal Management of Sickle Cell Anemia: Lessons from Nigeria". Anemia. https://doi.org/10.1155/2012/607436.

Cyril-Olutayo, M.C., Agbedahunsi, J.M., Akinola, N.O., 2019. Studies on the effect of a nutritious vegetable, *Telfairia occidentalis*, on HbSS blood. Jourl Tradnl Comple Medi. https://doi.org/10.1016/j.jtcme.2017.08.013.

Chikezie, P.C., Ekeanyanwu, R.C., Chile-Agada, A.B., 2020. Polymerization of deoxygenated sickle hemoglobin in the presence of fractionated leaf extracts of *Anacardium occidentale*, *Psidium guajava*, and *Terminalia catappa*. Bull Natl Res. https://doi.org/10.1186/s42269-020-00392-y.

Uysal, A., Ozer, O.Y., Zengin, G., Stefanucci, A., Mollica, A., Picot-Allain, C.M.N., Mahomoodally, M.F., 2019 Feb. Multifunctional approaches to provide potential pharmacophores for the pharmacy shelf: Heracleum sphondylium L. subsp. ternatum (Velen.) Brummitt. Comput Biol Chem. 78 (64–73). https://doi.org/10.1016/j.compbiolchem.2018.11.018.

Bibi Sadeer, N., Llorent-Martínez, E.J., Bene, K., Fawzi Mahomoodally, M., Mollica, A., Ibrahime Sinan, K., Stefanucci, A., Ruiz-Riaguas, A., Fernández-de Córdova, M.L., Zengin, G., 2019 Sep. Chemical profiling, antioxidant, enzyme inhibitory and molecular modelling studies on the leaves and stem bark extracts of three African medicinal plants. J Pharm Biomed Anal. 10 (174), 19–33. https://doi.org/10.1016/j.jpba.2019.05.041.

Sickle Cell Disease, 2021. https://www.cdc.gov/ncbddd/sicklecell/index.html.