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PHARMACEUTICAL AND MICROBIOLOGICAL STANDARDIZATION OF *SICULINE SYRUP*® FORMULATION, AN ANTISICKLING HERBAL MEDICINE

Uduak Ime Olayemi[a], Anthony Adebolu Elujoba[a], Ayobami Olutayo Oyedele[*b] and Oluwatoyin Abimbola Igbeneghu[b].

[a] Department of Pharmacognosy, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife Nigeria.
[b] Department of Pharmaceutics, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife Nigeria.

***Corresponding author E-mail:** aoyedele@oauife.edu.ng , uduakolayemi@gmail.com , tonyelu@yahoo.com,

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Abstract

Background: This study utilized 4:1 combination ratio of *Carica papaya* fruit mesocarp and *Sorghum bicolor* leaf fermented extract freeze-dried and named *Siculine extractive* (SE) as the active principle to develop *Siculine syrup* as an herbal formulation with potent antisickling properties.
Materials and Methods: I*n-vitro* antisickling (inhibitory or reversal) activities of test (SE) and control samples were determined on sodium metabisulphite-induced sickled red blood cells collected from confirmed non-crisis sickle cell patients. Particulate, pH and microbiological qualities of SE were determined toward its use in formulation.
The activities of SE aqueous dispersion (1-6 mg/ml) and of the formulated *Siculine syrup*® were evaluated using buffered normal saline (negative control), vanillic acid, parahydroxy benzoic acid (PHBA) and Ciklavit® (an herbal antisickling commercial product), as positive controls.
Results: The processed plant materials yielded 17.7±1.4 %w/w of water-insoluble, amber coloured particles (27.4 – 274.0 µm size range) of SE powder with microbiological quality suitable for oral liquid formulation. SE aqueous dispersion, neutral in pH, demonstrated concentration-related sickling inhibitory and reversal activities. The 5.0 mg/ml aqueous dispersion exhibited optimum antisickling potential namely, 80 % inhibitory and 66 % reversal effects, which were statistically equivalent to activities of the *Siculine syrup*® formulation, reference Ciklavit®, and 4.0-6.0 mg/ml PHBA's reversal activity, but higher than the inhibitory activity of 4.0-6.0 mg/ml vanillic acid.
Conclusion: *Siculine syrup*® formulation containing 10, 2, 0.5, and 0.25 %w/v of sucrose, tragacanth, SE, and parabens, respectively, demonstrated optimal physicochemical and microbiological stability properties with strong antisickling activities comparable to those of Ciklavit®.

Key words: *Carica papaya*, *Sorghum bicolor*, *Siculine extractive*, sickling inhibition, sickling reversal, syrup formulation.

Introduction

Sickle cell disease (SCD) affects large populations of the world particularly in the African and Asian continents, with an estimated figure of 200,000 people yearly (Dapa and Gil, 2002). It is a genetic blood disorder in which the red blood cells (RBCs) assume an abnormal, rigid sickle shape. Sickling decreases flexibility of the RBCs, resulting in a risk of various complications including anaemia. SCD occurs due to change (genetic mutation) of an amino acid within the beta globin chain of the hemoglobin molecule, whereby glutamic acid (a polar amino acid) is replaced by valine (a non-polar amino acid). At hypoxia (low oxygen tension in the narrow blood vessels), the mutant hemoglobin polymerizes within the RBCs into a gel or fibers leading to a drastic decrease in the red cell membrane deformability (Hartwell *et al*., 2000; Iyamu *et al*., 2003). Polymerization and precipitation of sickle hemoglobin (HbS) within the erythrocytes cause the change of shape from the normal spherical form into one resembling a sickle, hence the name sickle cell (Serjent, 2001). No permanent cure has yet been found for SCD but treatments can alleviate the symptoms and reduce complications that the patients often suffer, such as chronic anaemia, bone and joint pains, jaundice, hepato-splenomegaly, chronic infections, etc. In conventional management, infants diagnosed with SCD through newborn screening may be treated with antibiotics to prevent infections, blood transfusions commonly used to treat worsening of

anaemia and vaso-occlusive complications but then for economically buoyant patients, bone-marrow stem cell transplantation could be performed (Wu *et al*., 2006).

Majority of the populations in developing countries rely entirely on traditional medicine therapies based on plant extractives as their primary form of health care; medicinal plant recipes have provided relief for disease conditions pending discovery of conventional drugs for the management of many diseases (Sumner, 2000). As there is yet no promising orthodox drug known for SCD treatment and the high number of low-income people afflicted by the disease often cannot afford the cost of orthodox treatment, research has necessarily continued over the years in an effort to determine the efficacy of natural and artificial processes, including plant materials having anti-sickling effect. Extracts from many different medicinal plants that demonstrate *in vitro* or *in vivo* anti-sickle cell properties have been studied, and mini reviews of several plants potentially useful for the control of SCD symptoms have appeared in literature (Sahu *et al*., 2012; Nwaoguikpe, 2009; Okpuzor *et al*., 2008).

Much interest for research into SCD management with phytomaterials was generated by the serendipitous discovery of sickling inhibitory and reversal activities of the root extract of *Fagara zanthoxyloides* (Sofowora and Isaac-Sodeye, 1971), later renamed as *Zanthoxylum zanthoxyloides*, which led to many antisickling herbal remedies manufactured for the patients in the West African sub region, e.g. Ciklavit®, produced by Neimeth Pharmaceuticals Nigeria from the seed extract of *Cajanus cajan* (L.) Leguminosae-Fabaceae (Onah *et al*., 2002); Jobelyn® developed from *Sorghum bicolor* leaf extract to increase haemoglobin concentration, packed cell volume and white blood cell levels (Okochi *et al*., 2003; Okpuzor *et al*., 2008); and Hemodya® consisting of the stem-bark extracts of *Cassia siamea* Lam (Fabaceae), *Delonix regia* (Hook) Raf. (Fabaceae) and *Garcinia cowa* Roxb. (Clusiaceae), marketed in Cameroon (Agbor *et al*., 2015).

Following initial report of sickling inhibition and reversal properties of unripe *Carica papaya* Linn (Caricaceae) fruit aqueous extract (Thomas and Ajani, 1987), subsequent studies ascertained its safety (Oduola *et al*., 2007a) and determined its minimum concentration for activity in Wistar albino rats (Oduola *et al*., 2006); while pilot, oral use clinical trials demonstrated that it had no adverse effect on liver, kidney or haematological functions of clinical subjects (Oduola *et al*., 2007b; 2008; 2012). The *C. papaya* unripe fruit aqueous extract, when fermented over 5 days, exhibited comparatively higher antisickling potencies (87% inhibitory, 74% reversal activities) than when fermented for shorter or longer duration, and possessed higher effects than when methanol or chloroform was used as the extraction solvent (Ogunyemi *et al*., 2008). Equal weights of *C. papaya* unripe fruit extract and *Sorghum bicolor* (L.) Moench (Poaceae) leaf extract, when fermented together in water for 5 days (Cyril-Olutayo *et al*., 2009), demonstrated even greater antisickling activities (93 % inhibitory, 84 % reversal).

Continued research remains needful to discover more effective, affordable and available antisickling herbal recipes for prompt alleviation of the SCD symptoms in high-incidence populations, and to develop more remedies pending the discovery of permanent cure. Therefore, the aim of this study was to combine extractives of two plant parts: *C. papaya* unripe fruit mesocarp and *S. bicolor* leaf in a promising, more effective powder blend proportion (termed *Siculine extractive*) for use as a galenical to produce an effective oral liquid antisickling herbal medication called *Siculine syrup*, formulated with appropriate pharmaceutical excipients to have satisfactory physicochemical and microbiological stability qualities, and able to provide prompt relief for SCD symptoms.

This is the report of standardizing the composition, antisickling effects and stability attributes of *Siculine syrup*, an antisickling herbal medicine formulation, patented by this research group in the Federal Republic of Nigeria as a novel herbal medicine for the management of Sickle cell disease (Reference number NG/PT/NC/2017/2308, dated 27th June 2018).

Materials and Methods

Plant collection, authentication and processing

Matured, fresh unripe *Carica papaya* L. (Caricaceae) fruit was collected from Road 7, Obafemi Awolowo University (OAU) campus, Ile-Ife, Nigeria while *Sorghum bicolor* (L.) Moench (Poaceae) dried leaf was procured at the New Market, Ile-Ife, Nigeria. They were subsequently identified by the Plant Curator, Mr. G. Ibhamebhor of Botany Department, OAU, Ile-Ife and their voucher specimens deposited at IFE Herbarium OAU Ile-Ife, Nigeria under the reference numbers: IFE 17404 (*Carica papaya*) and IFE 17405 (*Sorghum bicolor*).

The greenish epicarp of the *C. papaya* fruits was peeled off, the inner whitish seeds discarded and the pulp (mesocarp) cut into small pieces, oven-dried at 50 °C and then powdered with a laboratory mill (Christy and Norris, England). The procured dried *S. bicolor* leaf was also similarly pulverized with the grinder. The powdered *C. papaya* fruit mesocarp and *S. bicolor* leaf were mixed together in a 4:1 ratio, respectively (considered more potent than 1:1 ratio used by Cyril-Olutayo *et al*.; 2009), and the combined powder (125.0 g) fermented in 3000 mL distilled water in a conical flask left undisturbed in the laboratory for five days at the ambient temperature (28±3 °C). The liquid galenical was then filtered using a Whatman No. 1 filter paper and the filtrate boiled for 1 h (thereby arresting the fermentation process and reducing microbial load). The resulting liquor was allowed to cool (≈ 28 °C) and was then freeze-dried to yield the dried *Siculine extractive* powder. The percentage yield of *Siculine extractive* powder was determined by comparing the weight of *Siculine extractive* powder obtained to the initial added weights of dried *C. papaya* fruit and *S. bicolor* leaf, in duplicate experiments.

Particulate, pH properties and microbiological quality determinations of *Siculine extractive* powder

A sample (0.2 g) of the *Siculine extractive* powder was examined under the microscope at ×100 magnification in order to observe its particulate features. The powder's particle size range was determined from calibration of the microscope lenses with a stage micrometer and an eye-piece graticule. Also, the pH profile of a 5 mg/mL dispersion of the *Siculine extractive* powder in freshly-prepared distilled water (i.e. 0.5 %w/v; 10 mL, duplicate aqueous dispersions) kept at 4 °C, 28 °C, and 38 °C in a thermo-regulated incubator, respectively, was determined with a digital pH meter (HM Digital, Inc. CA USA) over a 7-day storage period.

Furthermore, a 100 mg sample of the *Siculine extractive* powder was dispersed aseptically in 10 mL of sterile 0.1 % peptone water in a test tube to give a 1 in 100 w/v stock dispersion. Serial 1-in-10 dilution (v/v) of 1 mL aliquots of the stock *Siculine extractive* powder dispersion was then subsequently made in peptone water in test tubes, to produce a 1-in-10, 1-in-10^2, 1-in-10^3, and 1-in-10^4 dilutions of the stock dispersion. Then 0.5 mL of the stock dispersion and of each of its dilutions was plated (using sterile pipette) and spread (with a flamed and cooled glass spreader) on the surface of an over-dried Mueller-Hinton agar (MHA) plate, as well as on over-dried Sabouraud dextrose agar (SDA) plate, in duplicates, for viable count determination of recoverable bacterial and fungal colonies, respectively (Lamikanra, 2010). The plates thus inoculated were incubated at 37 °C for 48-72 h for bacterial growth and at 25±2 °C for 5 days for fungal growth. Following the incubation periods, colonies of the organisms present, found growing on the plates, were counted, for estimation of the microbiological quality of *Siculine extractive* powder.

Blood samples collection

Fresh whole blood samples (5 mL each) from confirmed sickle cell (HbSS) patients in steady state were collected at the Haematology Outpatient Clinic of Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC), Ile-Ife, Nigeria, by vein-puncture, into EDTA (ethylene diamine tetra acetic acid) bottles and used within 24 hours of collection. Ethical clearance for this procedure had been earlier obtained (Ref. No. ERC/2016/09/19) from the OAUTHC Research Ethics Committee, and prior informed consent of each volunteer (patient) that participated in the study was appropriately obtained.

Inhibitory antisickling assay

The method described by Cyril-Olutayo *et al.* (2009) was used: A sample of the HbSS whole blood (0.2 mL) was pipetted into a clean test tube (in duplicates) containing 0.2 mL of phosphate buffered normal saline (pH 7.0). To this was added and gently mixed together, the following test or control samples, each in sequential experiments, respectively: 0.2 mL aqueous dispersion of *Siculine extractive* powder (active principle under investigation) or vanillic acid (Sigma-Aldrich, as positive control) each at 1, 2, 3, 4, 5, and 6 mg/mL concentrations, respectively; or 0.2 mL of *Siculine syrup* formulation (later described), or of phosphate buffered normal saline (pH 7.0, as negative control), or of Ciklavit® (a commercial antisickling herbal product FDA-listed in Nigeria; another positive control). The mixture was overlaid with liquid paraffin (1 mL) and incubated in a thermostated water bath (37 °C) for 4 h. Thereafter, 0.6 ml of a 2 %w/v sodium metabisulphite aqueous solution was carefully added, using a Pasteur pipette, underneath the liquid paraffin overlay in the test tube. This reaction mixture was thoroughly mixed by gently rolling the test tube between the palms and incubated for another 1½ h in the water bath (37 °C).

At the end of the incubation period, the liquid paraffin overlay was carefully removed using a Pasteur pipette. Then 3 mL of a 5 % buffered formalin solution (pH 7.0) was added to the reaction mixture that remained in the test tube in order to fix the red blood cells. A drop of the sample was then placed on a glass slide, covered carefully with a cover slip and examined under the microscope at ×400 magnification. The numbers of sickled and unsickled cells in five different fields of view were counted and recorded.

Reversal antisickling assay

The method of Cyril-Olutayo *et al.* (2009) was used: A sample of the HbSS whole blood (0.2 mL) was pipetted into a test tube (in duplicates) into which 0.2 mL of phosphate buffered normal saline (pH 7.0) had been placed, and mixed. Liquid paraffin (1 mL) overlay was added to this mixture and 0.6 mL of sodium metabisulphite solution (2 %w/v) was carefully introduced with a Pasteur pipette underneath the liquid paraffin overlay, followed by incubation in a water bath (37 °C) for 1½ h. At the end of the incubation period, 0.2 mL of the following test or control agents was added each in sequential experiments, respectively, and mixed carefully as before: *Siculine extractive* powder (aqueous dispersion) or para-hydroxy benzoic acid (PHBA; positive control) each at 1, 2, 3, 4, 5, and 6 mg/mL concentrations, respectively; *Siculine syrup* formulation (later described); phosphate buffered normal saline (pH 7.0; negative control); or Ciklavit® (positive control); followed by incubation in water bath (37 °C) for another 6 h.

At the end of the latter incubation period, the liquid paraffin overlay was removed and 3 mL of 5 % buffered formalin solution (pH 7.0) was added to the reaction mixture in the test tube to fix the red blood cells. A drop of the reaction mixture was then placed on a slide as before, covered with a cover slip and examined under the microscope at

×400 magnification. The numbers of sickled and unsickled cells in five different fields of view in each case were counted and recorded.

The percentage inhibitory and/or reversal antisickling activities in both the test and control experiments were calculated according to the following formulae (Cyril-Olutayo *et al.*, 2009):

$$\% \text{ Sickled cells} = \frac{\text{Number of sickled cells}}{\text{Total number of sickled and unsickled cells}} \times 100\ \% \quad (1)$$

$$\% \text{ Inhibitory or Reversal activity} = \frac{a - b}{a} \times 100\ \% \quad (2)$$

where: a = % sickled cells in the negative control sample; and b = % sickled cells in the test sample (or in positive control)

Preparation of *Siculine syrup* and stability study

A stable preparation of *Siculine syrup* containing 0.50 %w/v of *Siculine extractive* powder (being the active herbal principle) was formulated as follows (Table 1): *Siculine syrup* with the parabens (methyl and propyl parabens blend of 0.20 and 0.05 %w/v, respectively) as preservative, sucrose as sweetener, tragacanth as suspending or viscosity imparting agent, and Water for Preparations BP (British Pharmacopoeia, 2009) as the continuous (aqueous) medium.

Table 1: Composition of *Siculine syrup*® formulation

Ingredient	Concentration
Siculine extractive powder	0.50 g
Methyl paraben	0.20 g
Propyl paraben	0.05 g
Sucrose	10.00 g
Tragacanth	2.00 g
Water BP	to 100 mL

Physicochemical stability and antimicrobial preservative efficacy studies

Physicochemical stability of *Siculine syrup* was evaluated using the viscosity, sedimentation profile and pH of the prototype products, containing graded tragacanth concentrations, monitored over 6 weeks, while the antimicrobial preservative efficacy determination was carried out on *Siculine syrup* formulation that demonstrated optimum physical stability. Viscosity was determined using the Ostwald U-tube viscometer, sedimentation volume ratio (obtained by the standard formula: F = Vu/Vo; where Vu and Vo are the volume of sediment and total volume of the preparation, respectively) determined in duplicate, in 25-ml stoppered calibrated glass cylinders at room temperature (28±3 °C); pH was determined using the HM digital pH meter, and preservative efficacy testing carried out against the specified challenge organisms, namely: *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus aureus* (NCTC 6571), *Candida albicans* (an isolate obtained at OAUTHC), and *Aspergillus niger* (an environmental isolate obtained from Microbiology Department, OAU Ile-Ife, respectively, in accordance with the BP (2009) procedure.

Data analysis

The results of determinations carried out in duplicate or triplicate were expressed as mean ± SD or mean±SEM, respectively. The latter were subjected to two-way analysis of variance (ANOVA), and P values less than 0.05 ($p<0.05$) were regarded as statistically significant.

Results

Particulate, bulk properties and yield of *Siculine extractive* powder

Siculine extractive powder consisted of irregularly shaped (amorphous), dark brown particles of 27.4 to 274.0 µm size range, most of which exhibited rapid sedimentation (within 15 seconds) when dispersed in freshly distilled water (showing particles of greater density than 1.0). In bulk (macroscopic) features, the dark brown (or amber) coloured *Siculine extractive* powder was mostly coarse; while its neat, aqueous dispersion or other simple aqueous preparation (e.g. mixture, suspension) of the powder immediately assumed its amber colour upon constitution.

The yield of *Siculine extractive* powder, expressed as %w/w of the starting plant materials (the unripe *C. papaya* fruit pulp powder and *S. bicolor* leaf powder), was 17.71±1.38 %w/w.

Microbiological quality of *Siculine extractive* powder and pH stability of its aqueous dispersion

The microbiological quality evaluation of *Siculine extractive* powder showed that it contained 3.7×10^3 colony forming units per gram (cfu/g) of bacterial cells and 1.0×10^3 cfu/g of fungal cells being the total viable count values determined, respectively. These microbial load levels of the crude galenical were satisfactory in reference to the World Health Organization (WHO) guidelines (2007) and the USP XVII limits for microbial contaminants in substances of vegetable origin for use in pharmaceutical production (WHO, 2007; Rawlings, 1980).

The 0.5 %w/v aqueous dispersion of *Siculine extractive* powder exhibited nearly neutral pH (5.1–7.6), with only slight variations of 1.3–2.5 units occurring in the pH value of the freshly prepared dispersion over 7 days at different storage temperatures, 4 °C through 38 °C (Table 2).

Antisickling activities of *Siculine extractive* powder in aqueous dispersion

Siculine extractive (SE) powder aqueous dispersions, at 1 – 6 mg/mL concentrations, demonstrated concentration-dependent inhibitory antisickling activities of 23.16 – 83.66 % (Figure 1). At different test concentrations: 1, 2, 3, 4, 5, and 6 mg/mL, the vanillic acid (VA)-equivalent inhibitory antisickling activity (IAA) values of the SE powder activities were: 5.7, 4.3, 2.6, 1.5, 1.3, and 1.2, respectively; derived from Figure 1 data as the quotient (value) of SE activities versus VA activities (Ajayi *et al*., 2020). Thus, SE powder exhibited greater inhibitory antisickling activities than the positive control, vanillic acid, at all the test concentrations (Figure 1). The strong (≥ 80%) inhibitory activities of the 4.0, 5.0 and 6.0 mg/mL SE powder concentrations were statistically comparable, and equivalent also to Ciklavit® activity (80.52±5.36 %). The Ciklavit®-equivalent IAA activity of SE activity was 1.0 (by same computation as above).

SE powder aqueous dispersions at 1 – 6 mg/mL concentrations also demonstrated concentration-dependent reversal antisickling activities of 3.60 – 64.73 % (Figure 2). At different test concentrations: 1, 2, 3, 4, 5, and 6 mg/mL, the PHBA-equivalent reversal antisickling activity (RAA) values of the SE powder activities were: 0.3, 0.4, 0.6, 1.0, 1.0, and 1.0, respectively; derived similarly from Figure 2 data as the quotient RAA values of SE versus PHBA activities (Ajayi *et al*., 2020). Thus, the relatively high reversal antisickling activities (61-66%) of SE powder at 4.0, 5.0 and 6.0 mg/mL concentrations (Figure 2) were equivalent to those of PHBA (positive control agent), as well as statistically comparable and equivalent to that of Ciklavit® activity (67.85±7.50 %). The Ciklavit®-equivalent RAA activity value of SE activity was also 1.0.

The overall inference from these analyses of the results (Figures 1 and 2) was that *Siculine extractive* powder at 5.0 mg/mL in aqueous suspension gave the optimal inhibitory and reversal antisickling activities, and would therefore be appropriate and suitable for syrup formulation.

Table 2: pH profile of 0.5 %w/v aqueous dispersion of *Siculine extractive* powder in different storage conditions over 7 days

	Storage temperature (°C)/ pH value* of *Siculine extractive* powder dispersion		
Time (Days)	**4 °C (Refrigeration)**	**29±2 °C (Room temperature)**	**38 °C (Warm Condition)**
0	5.1	5.1	5.1
1	5.6	5.1	5.5
2	5.6	5.1	5.5
3	5.9	6.9	6.1
4	5.7	6.7	6.4
5	6.0	7.2	7.3
6	6.1	7.5	7.6
7	6.4	7.6	7.6
Difference in pH over storage period:	**1.3**	**2.5**	**2.5**

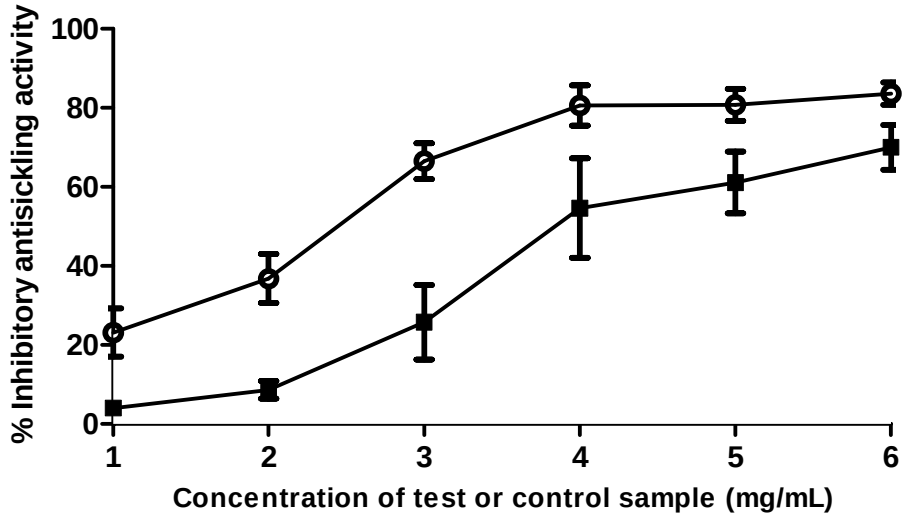


Figure 1: Inhibitory antisickling activities of Siculine extractive powder and Vanillic acid aqueous dispersions at different concentrations

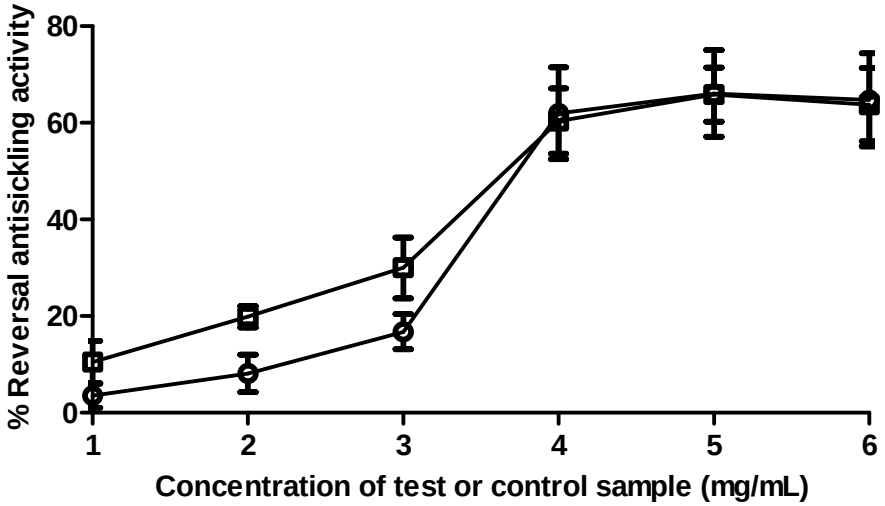
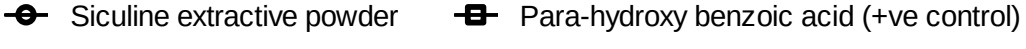


Figure 2: Reversal antisickling activities of Siculine extractive powder and Para-hydroxy benzoic acid (PHBA) aqueous dispersions at different concentrations



Inhibitory and reversal antisickling activities of *Siculine syrup*®

Freshly prepared *Siculine syrup*® demonstrated strong inhibitory antisickling activity (≈82 %) that was equivalent to the inhibitory activity of Ciklavit® (Table 3) and comparable to the inhibitory antisickling activities of 4.0–6.0 mg/ml *Siculine extractive* powder aqueous dispersion: 80-83% (Figure 1). Likewise the freshly prepared *Siculine*

syrup® formulation also demonstrated considerable reversal antisickling activity (≈65%), comparable to the reversal activities of *Siculine extractive* powder (5.0, 6.0 mg/mL aqueous dispersion) with ≈65 %, and also comparable to that of Ciklavit® with ≈68 % (Table 3). The inhibitory and reversal antisickling activities of the *Siculine syrup*® were not significantly altered (or reduced) during initial 6 weeks shelf life of the formulation (Table 3).

***Siculine syrup*® stability properties**

Siculine syrup composition (Table 1) produced a suspension (solid particulate dispersion) formulation with tragacanth as its physical stability (consistency and viscosity) modifier. The initial 7 days' dynamic viscosity data of *Siculine syrup* pre-formulation prototypes (A – E) containing five ranked tragacanth concentrations (0.2, 0.5, 1.0, 1.5, and 2.0 %w/v) in Table 4, and the 6-week storage-time monitored viscosity data of the optimal formulation (coded E) as in Figure 3, along with their sedimentation profile over the study period (Table 5), revealed the physical stability characteristics of the *Siculine syrup* formulation.

At times during the 6-week storage period, the *Siculine syrup* formulation prototypes A through C (containing tragacanth at 1.0 % or lower concentrations) exhibited sedimentation volume ratios of significantly less than 1.0 (Table 5). All these prototypes, however, remained re-dispersible (by shaking the container) throughout storage period. On the whole, the viscosity (Table 4) and sedimentation ratio values generally increased with increase in concentration of the viscosity modifier present as expected, until the maximum (100 %) sedimentation ratio value of "1.00" was attained in the *Siculine syrup* C, D and E formulations (Table 5).

The overall best prototype was the *Siculine syrup* formulation E that contained 2 % tragacanth, which gave absolutely non-sedimenting products (Table 7). The *Siculine syrup*® formulation (E) at the ambient temperature maintained a high viscosity value (≥131 cP) for 5 weeks (desirable and useful for repeated, uniform dose dispensing of a pourable liquid product) before a decline occurred (Figure 3). The viscosity decline that occurred beyond the 5th week of storage indicated an exhaustion of the stabilizing strength of the gel network structure of the suspending agent.

Table 6 presents the pH profile of *Siculine syrup*® (prototype E formulation) under different storage temperatures, showing that only minimal change occurred in the approximately neutral pH values over 6-week storage.

Results of preservative efficacy testing of best *Siculine syrup* formulation (prototype E)

The preservative system of *Siculine syrup*® (prototype formulation E) consisting of methyl and propyl parabens blend (0.20 and 0.05 %w/v, respectively; Table 1) produced: 2.6, 1.9, 3.0 and 1.6 log reduction in 48 h; and 3.2, 3.1, 3.7, and 3.9 log reduction in 14 days, in the viable counts of the challenge organisms: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger*, respectively, with no increase in the counts thereafter (Figure 4). These results satisfied the criteria for effective preservation of oral products made with aqueous vehicles (Pharmaceutical Microbiology Manual 2014, published in 2015).

Table 3: Inhibitory and reversal antisickling activities of *Siculine syrup*®

	Parameters of Antisickling assay/ % Inhibitory or Reversal antisickling activities* of *Siculine syrup*®			
***Siculine syrup*$^{(R)}$ storage duration (weeks)**	**% Inhibitory activity of *Siculine syrup*®**	**Ciklavit®-equivalent (×*f*) for *Siculine syrup*® inhibitory activity¥¥**	**% Reversal activity of *Siculine syrup*®**	**Ciklavit®-equivalent (×*f*) for *Siculine syrup*® reversal activity¥¥**
0	82.09 ± 3.85[a]	1.02	64.64 ± 6.02[b]	0.95
2	78.58 ± 3.76[a]	0.98	64.61 ± 6.27[b]	0.95
6	79.69 ± 2.77[a]	0.99	66.05 ± 5.58[b]	0.97

Key:
* Data are expressed as Mean ± SEM.
¥¥ "Ciklavit®-equivalent (×*f*) for *Siculine syrup*® inhibitory (or reversal) activity" i.e. Values indicating the factor ("*f*") by which to multiply the Ciklavit® inhibitory or reversal activity, to obtain the activity of *Siculine syrup*$^{(R)}$ inhibitory or reversal activity, respectively.
[a] p>0.05 for all values so indicated, which also are not significantly different compared to Ciklavit® inhibitory activity.
[b] p>0.05 for all values so indicated, which also are not significantly different compared to Ciklavit® reversal activity.

Table 4: Dynamic viscosity profiles of prototype *Siculine syrup* formulations

Storage Time (Days)	Code (A-E) of prototype *Siculine syrup* formulations; Tragacanth (viscosity modifier) content (%w/v)/ Dynamic viscosity of the formulation (centi Poise; cP)*				
	A; 0.2 %	**B; 0.5 %**	**C; 1.0 %**	**D; 1.5 %**	**E; 2.0 %**
1	2.65 ± 0.04^{a}	5.85 ± 0.08^{c}	21.96 ± 0.16^{d}	56.71 ± 0.19^{e}	132.26 ± 0.17^{f}
2	2.63 ± 0.05^{a}	5.96 ± 0.10^{c}	21.97 ± 0.28^{d}	56.76 ± 0.18^{e}	132.02 ± 0.43^{fg}
3	2.60 ± 0.03^{ab}	5.97 ± 0.07^{c}	22.13 ± 0.21^{d}	56.75 ± 0.16^{e}	131.71 ± 0.37^{fgh}
4	2.64 ± 0.02^{a}	6.01 ± 0.02^{c}	22.06 ± 0.32^{d}	56.69 ± 0.28^{e}	131.82 ± 0.59^{fgh}
5	2.58 ± 0.02^{b}	5.98 ± 0.08^{c}	22.04 ± 0.14^{d}	56.63 ± 0.28^{e}	131.61 ± 0.40^{fgh}
6	2.56 ± 0.03^{b}	5.96 ± 0.10^{c}	21.99 ± 0.14^{d}	56.78 ± 0.28^{e}	131.88 ± 0.33^{fgh}
7	2.66 ± 0.04^{a}	5.92 ± 0.11^{c}	21.90 ± 0.19^{d}	56.72 ± 0.28^{e}	131.46 ± 0.60^{fgh}

Key:
* Data are expressed as Mean ± SEM
$^{a-h}$ Superscript letters: Values with same letters indicate non-significance of difference in the dynamic viscosity values, i.e. $p>0.05$.

Table 5: Sedimentation volume ratios of prototype *Siculine syrup* formulations

Storage Time (Days)	Code (A-E) of prototype *Siculine syrup* formulations; Tragacanth (viscosity modifier) content (%w/v)/ Sedimentation volume ratio of formulation.				
	A; 0.2 %	**B; 0.5 %**	**C; 1.0 %**	**D; 1.5 %**	**E; 2.0 %**
0	0.13	0.33	1.00	1.00	1.00
1	0.09	0.21	1.00	1.00	1.00
2	0.09	0.21	1.00	1.00	1.00
4	0.09	0.21	1.00	1.00	1.00
6	0.08	0.19	0.95	1.00	1.00
10	0.08	0.18	0.88	1.00	1.00
14	0.08	0.17	0.84	0.98	1.00
21	0.07	0.16	0.77	0.98	1.00
28	0.07	0.14	0.70	0.97	1.00
35	0.07	0.14	0.63	0.95	1.00
42	0.07	0.14	0.53	0.92	1.00

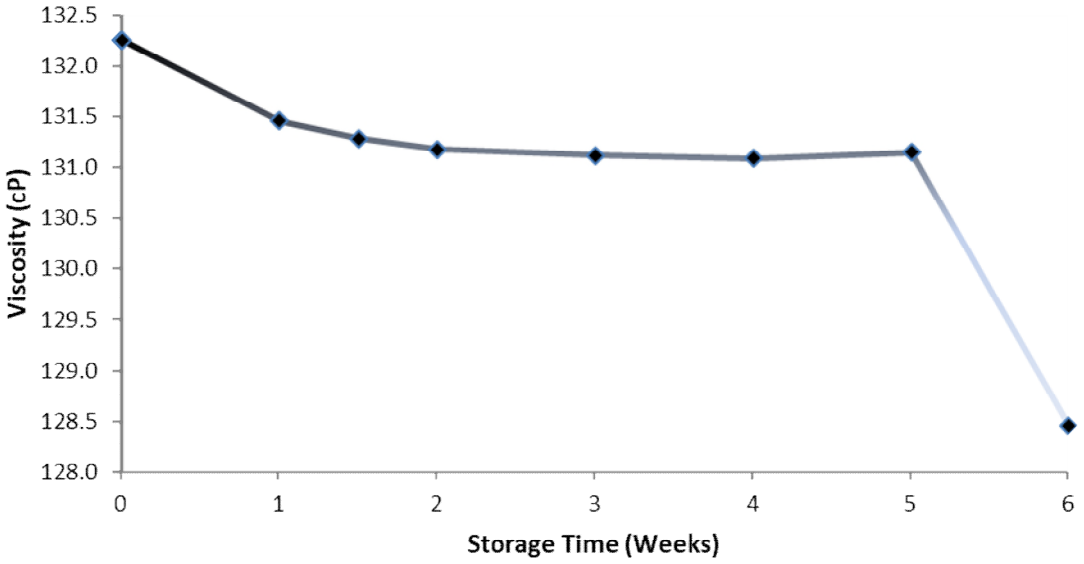


Figure 3: Dynamic viscosity profile of optimal *Siculine syrup* formulation (code E) over 6 weeks storage

Table 6: pH profile of prototype *Siculine syrup* formulation E under different storage temperatures

	Storage temperature (°C)/ pH value* of *Siculine syrup* formulation E**		
Time (Days)	**4°C (Refrigeration)**	**29±2°C (Room temperature)**	**38°C (Warm storage)**
0	6.5	6.5	6.5
1	6.9	6.7	6.5
2	6.7	6.5	6.3
3	6.9	6.6	6.5
4	7.1	6.7	6.3
5	6.7	6.8	6.5
6	7.0	6.7	6.5
7	7.8	6.9	6.7
14	7.4	7.3	7.0
21	7.1	6.8	6.5
28	7.3	6.4	6.2
35	7.0	6.3	6.3
42	6.8	6.4	6.2

Key:
* The precision of pH determinations was within approx. ±0.5 of the value obtained
** Same composition as Table 1; containing 2.00 %w/v of tragacanth.

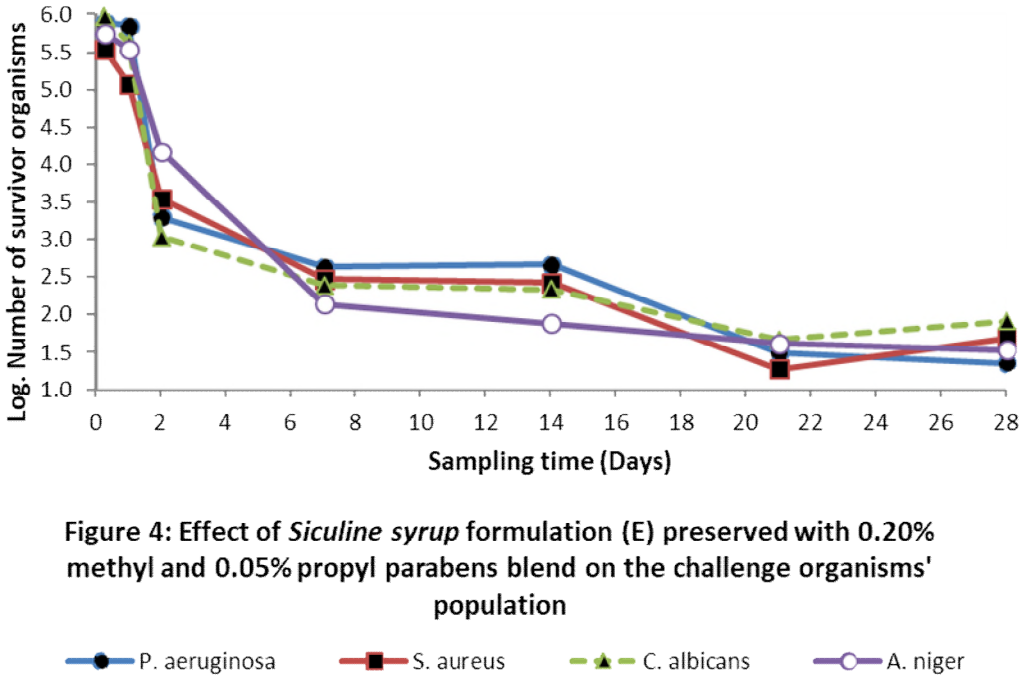


Figure 4: Effect of *Siculine syrup* formulation (E) preserved with 0.20% methyl and 0.05% propyl parabens blend on the challenge organisms' population

Discussion

This study reports particulate characteristics, microbiological qualities and antisickling activities of *Siculine extractive* powder, the active herbal principle of the syrup formulation. In the study, the antisickling (inhibitory and reversal) activities of *Siculine extractive* powder, consisting of optimal-ratio (4:1) mixture of *C. papaya* fruit powder and *S. bicolor* leaf powder respectively, have been exploited for pharmaceutical formulation of *Siculine syrup*. Both the formulation and its active principle (galenical) have been evaluated for antisickling activities in parallel experiments along with a previously FDA-listed herbal supplement for SCD management, Ciklavit®, and also with the universal antisickling chemical agents, vanillic and p-hydroxy benzoic acids (Ogunyemi *et al*., 2008) as positive controls. The packaging label information on Ciklavit® with Lot number: 50836030A; FDB/SD. 05-12706 states that its active principle is *Cajanus cajan* extractive (40 mg/10 mL, calculated as total protein) along with ascorbic acid and zinc (50 mg and 400 ppm per mL, respectively). Ekeke and Shode (1990) reported *Cajanus cajan* seed to contain phenylalanine as its predominant antisickling agent which is also present in *C. papaya* fruit among others (including aspartic acid, glutamic acid and lysine) (USDA, 2009; Duke 1992). These amino acids may therefore be partly responsible for the observed antisickling activities of *C. papaya* fruit pulp in its combination with the *S. bicolor* leaf, as active components of *Siculine syrup*®, which demonstrated potent activities comparable to Ciklavit® (Table 3).

Thus, this study has fulfilled our aims of formulating bioactive plant extractives, using pharmaceutical excipients of suitable characteristics to produce a standardised herbal medicine of acceptable therapeutic potency. This culminates in finished product that exhibits reproducible chemical profile and consistent pharmacological activities, which ensures minimal batch-to-batch variations in quality, efficacy and safety (Neeraj and Bhupinder, 2011; Archana *et al*., 2010).

The results of comparative antisickling activities in this study have revealed, on the one hand, that *Siculine extractive* aqueous dispersion and PHBA, each at 4.0 – 6.0 mg/mL concentrations, exhibited similar sickling reversal activities (60-66%), which were equivalent to those of Ciklavit® (Figure 2); and that similar antisickling inhibitory activities (≥80%) were demonstrated by *Siculine extractive* dispersions (4.0 – 6.0 mg/ml) and Ciklavit®, which were higher than the inhibitory activities of vanillic acid at 4.0 – 6.0 mg/mL concentrations (Figure 1). The comparative antisickling activity results also revealed, on the other hand, that Ciklavit® and *Siculine syrup*® demonstrated equivalent antisickling (inhibitory and reversal) activities (Table 3). The recommended dose of Ciklavit® for use by children above 5 years old and adults is ≈30 mL; and is 10 mL dose for children below 5 years old, twice daily. In view of the antisickling potency equivalence to that of *Siculine syrup*®, this dose regimen may be considered suitable for *Siculine syrup*®. Furthermore, the cold storage instruction (<25 °C, preferably refrigerated) is recommended on Ciklavit® packaging label, and coincidentally in the present study, the cold storage condition was shown to similarly promote physicochemical stability of *Siculine syrup*® and *Siculine extractive* powder, its precursor galenical (Tables 2 and 6), as is commonly the case for most liquid medications.

In addition to its potent antisickling activities, *Siculine syrup*® formulation demonstrated suitable physical stability properties, typically by the D and E prototypes containing 1.5 and 2 %w/v of tragacanth as viscosity modifier, respectively (Tables 4 and 5). Both concentrations of the suspending agent resulted in formulations of adequate and comparable physical stability, with negligible difference between their stability properties (Tables 4 and 5), and may be considered as alternative stable formulations for *Siculine syrup*®. Furthermore, satisfactory microbiological quality of *Siculine extractive* powder aqueous dispersion as well as the efficiency of the preservative system used in *Siculine syrup*® (Figure 4) were also demonstrated in the study.

Since the pH value of *Siculine syrup*® or of *Siculine extractive* powder aqueous dispersion would, expectedly, reflect the chemistry of the total ionic environment (consisting of several chemical or biochemical components) within the liquid system, the considerable stability observed of the pH profile for both the *Siculine extractive* in aqueous dispersion (Table 2) and the *Siculine syrup*® (Table 6) revealed considerable chemical stability of the aqueous liquid systems. The chemical stability of *Siculine syrup*® is concomitantly buttressed by the 5- and 6-week long visco-stability (Figure 3) and physical consistency (prototype E; Table 5), respectively, of *Siculine syrup*®.

Conclusion

Fresh, unripe *Carica papaya* mesocarp extract combined with *Sorghum bicolor* leaf extract in 4:1 proportions, respectively, and processed into *Siculine extractive* galenical, at 5 mg/mL aqueous dispersion, exhibited remarkable antisickling (reversal and inhibitory) effects on sodium metabisulphite sickled HbSS patients' erythrocytes when incubated together under simulated body temperature condition (37 °C). Consequently, *Siculine syrup*® has been developed in this study using *Siculine extractive* powder (with appropriate pharmaceutical excipients), as an herbal suspension formulation with potent antisickling properties for oral administration. The standardized *Siculine syrup*® composition, physical, chemical and microbiological stability characteristics, and antisickling activities are hereby reported.

Ethics approval

This project was approved by the Institutional Ethics Committee: Reference No. OAUTHC ERC/2016/09/19

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Conflict of Interest Declaration: None

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