Formulation and antisickling activity of the effervescent granules of *Cissus populnea* Guill et. Perr (Vitaceae) root on deoxygenated drepanocytes

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ABSTRACT

Background: Ethanol extracts of *Cissus populnea* have been documented to possess antisickling activities, useful in the management of Sickle Cell Anaemia (SCA). However, it is necessary to develop the extracts into acceptable dosage forms with further potentials for drug development.

Objectives: This present study investigated the in-vitro evaluation of the antisickling activities of the crude ethanol extract and effervescent granules of *Cissus populnea* roots.

Methods: *Cissus populnea* root was dried at 37±0.5^{o}C, powdered, extracted in 80 % ethanol (72 h.), filtered, concentrated, freeze-dried and prepared as effervescent granules (CPG) formulations 1, 2 and 3 with varying percentages of sodium bicarbonate, citric and tartaric acids, while the crude extract was formulation 4. CPG were characterized using density measurements (Bulk and Tapped Densities), Hausner's ratio [HR], Carr's Index [CI]) and angle of repose as assessment criteria, while antisickling properties were evaluated using inhibitory and reversal activities of sodium metabisulphite-induced sickling of hemoglobin erythrocytes. Para-hydroxybenzoic acid (PHBA) and phosphate-buffered saline solutions served as controls.

Results: Bulk and tapped densities ranked formulations 1(1.37±0.03) >3(1.18±0.13) >2(1.13±0.11)>4(1.07±0.07), and 3(1.69±0.03)>1(1.68±0.02) >2(1.51±0.07) >4(1.31±0.13) respectively. The HR and CI had a reverse relationship with the crude extract granules having the highest HR (1.03±0.53) and lowest CI (18.32±0.03) values. Angle of repose ranked formulations 4(39.18±0.11)>3(34.18±0.28)>2(31.03±0.27)>1(29.13±0.17). Formulations 3 and 4 showed the highest antisickling (72.0 % and 68.0%) and reversal activities (57.0 % and 59.0 %) comparable to PHBA (64 % inhibitory and 56.0 % reversal activities)

Conclusion: CPG showed better flow and antisickling properties than those of the crude extract, and may play important roles in the management of SCA.

Keywords: Sickle cell anemia, *Cissus populnea* Guill et. Perr ., Para-hydroxybenzoic acid (PHBA), Effervescent granules.

Formulation et activité antifalciforme des granules effervescents de la racine de Cissus populnea Guill et. Perr (Vitaceae) sur drépanocytes désoxygénés

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RÉSUMÉ

Contexte: Il a été démontré que les extraits d'éthanol de *Cissus populnea* possèdent des activités antifalciformes, utiles dans la gestion de l'anémie falciforme. Cependant, il est nécessaire de développer les extraits sous des formes posologiques acceptables, avec d'autres potentiels pour le développement de médicaments.

Objectifs: Cette étude a examiné l'évaluation in vitro des activités antifalciformes de l'extrait d'éthanol brut et des granules effervescents de racines de *Cissus populnea.*

Méthode: La racine de *Cissus populnea* a été séchée à 37±0,5ºC, réduite en poudre, extraite dans de l'éthanol à 80% (72 h), filtrée, concentrée, lyophilisée et préparée sous forme de granulés effervescents (CPG) formulations 1, 2 et 3 avec des pourcentages variables de bicarbonate de sodium, d'acides citrique et tartrique, tandis que l'extrait brut constituait la formulation 4. Les CPG ont été caractérisés à l'aide de mesures de densité (densités apparentes et tassées), du rapport de Hausner [HR], de l'indice de Carr [IC]) et de l'angle de repos comme critères d'évaluation, tandis que les propriétés antifalciformes ont été évaluées à l'aide des activités d'inhibition et d'inversion de la falciformation des érythrocytes de l'hémoglobine induite par le métabisulfite de sodium. L'acide para-hydroxybenzoïque (PHBA) et les solutions salines tamponnées au phosphate ont servi de témoins.

Résultats: Les densités apparentes et tassées ont classé les formulations 1(1,37±0,03) >3(1,18±0,13) >2(1,13±0,11) >4(1,07±0,07), et 3(1,69±0,03)>1(1,68±0,02) >2(1,51±0,07) >4(1,31±0,13) respectivement. Le HR et le CI avaient une relation inverse avec les granulés d'extrait brut ayant les valeurs HR les plus élevées (1,03±0,53) et CI les plus basses (18,32±0,03). L'angle de repos a classé les formulations 4(39.18±0.11)>3(34.18±0.28)>2(31.03±0.27)>1(29.13±0.17). Les formulations 3 et 4 ont montré les activités antifalciformes les plus élevées (72,0% et 68,0%) et d'inversion (57,0 % et 59,0 %), comparables à celles du PHBA (64 % d'activité inhibitrice et 56,0 % d'activité d'inversion).

Conclusion: Le CPG a montré de meilleures propriétés d'écoulement et antifalciformes que celles de l'extrait brut et peut jouer un rôle important dans la gestion de l'anémie falciforme.

Mots clés: Anémie falciforme, *Cissus populnea* Guill et. Perr., acide para-hydroxybenzoïque (PHBA), granulés effervescents.

INTRODUCTION

The use of herbal products (herbs) for the treatment of patients with sickle cell diseases (SCD) is a typical practice particularly in the provincial parts of West Africa where SCD is endemic. A prototype of such product is NIPRISANTM, a herbal drug manufactured by the National Institute for Pharmaceutical Research and Development (NIPRD), and generally being used in Nigeria, India and the United States of America, which has demonstrated an extraordinary indication for therapy.[1,2]

SCD is a hereditary abnormality caused by the advent of sickled hemoglobin. Usually, the red blood corpuscles (RBC) changes shape upon deoxygenation and hemoglobin polymerization into a reversible or irreversible sickle shape. The hemoglobin proteins adhere to each other, making the RBC have an unbending surface and sickle shape, and hence making the cells get stuck in the veins. This denies the downstream tissues of oxygen and causes ischemia and localized necrosis. Sickle cell anaemia is a hereditarily acquired illness in which the 'SS' homozygous individual has an unusual β-globin chain. A single base substitution in the gene encoding the human β-globin subunit brings about substitution of β6 glutamic acid by valine, prompting the different clinical signs of SCD. This substitution causes an exceptional decrease in the solvency of sickle cell hemoglobin (HbS) when deoxygenated.[3] Under these conditions, the HbS atoms polymerise to frame a long crystalline intracellular mass of fibers that are responsible for the disfiguration of the biconcave discoid erythrocyte into a sickle shape.

The plant *Cissus populnea* Guill et. Perr (Family: Vitaceae) has been documented as one of the major components of herbal formulations indicated for RBC sickling that are manufactured in Nigeria. The major phytochemicals present in *Cissus populnea* were analysed to be majorly anthraquinone derivatives, steroidal glycosides, and cardiac glycosides.[4] *Cissus populnea* is a plant with a series of medicinal uses in various parts of the world. Its concentrated extracts have been found to demonstrate antibacterial properties[5] and antisickling properties when incorporated in formulations.[4] In Benin Republic, it is utilized for its diuretic properties, while in Ghana it is utilized as a post-harvest ethnobotanical protectant.[6] It can also be administered as a fertility enhancer among males with erectile dysfunction.

In recent times, formulation scientists have been exploiting ways of developing natural extracts into dosage forms that have potential for further drug developments. Granulation is one of the most widely used applications in drug manufacturing processes and pharmaceutical granules have been used extensively in drug delivery when filled in capsule shells, as ingredients for tablets or for immediate constitution formulations.[7] The inclusion of citric acid, tartaric acid and sodium bicarbonate powder as part of the ingredients in the granulation process has been documented to enhance the absorption of the active ingredient and also ensure patient compliance with medications, due to the enhanced palatability that the ingredients confer on the final preparation, especially for formulations that will be reconstituted prior to administration.[8] This work presents an update on our previous study 4 by assessing and evaluating the antisickling potentials of effervescent granules of ethanol extract obtained from *Cissus populnea* root.

MATERIALS AND METHODS

Plant collection and preparation:

The plant of *Cissus populnea* was collected from the Molete region of Ibadan, Oyo State, South-Western Nigeria and authenticated at the Forest Herbarium Ibadan, Nigeria (FHI), with assigned voucher specimen number FHI-112268 The dried roots were pulverized, and stored in air and moisture-tight containers. All the other ingredients used as described were all of analytical grade.

Preparation of plant root extracts:

The root of the fresh plant was harvested, washed in purified water and dried at 37±0,5^{o}C (The temperature was monitored using a thermometer placed in the same room), until a uniform weight consistency was obtained. The dried root was then milled using a locally fabricated manual grinding machine before transferring to a planetary milling equipment (Retsch PM 400 MA, Retsch, Haan, Germany). The powdered root sample was macerated in 80 % ethanol for 72 h. The solution was decanted and filtered. The residue was re-soaked into the same solvent for another 72 h for exhaustive extraction. The filtrate was concentrated under reduced pressure at 35^{o}C using a rotatory evaporator (Germany Stuart Model 46A25: Speed range: 20 - 190 rpm). The concentrate was lyophilized using a freeze-drier (Freeze dryer Christ Alpha 1-2 LD, made in Germany).

Formulation of herbal effervescent granules: Effervescent granules of *Cissus populnea* root extracts were prepared by wet granulation method. Four different formulations of the granules were made in triplicates.

Formulations 1, 2 and 3 contained freeze-dried extracts of *Cissus populnea* root (FDCPR), citric acid granules, tartaric acid pellets and sodium bicarbonate, but in different proportions, while the fourth formulation contained only FDCPR as shown in Table 1. The concentration of each formulation was chosen based on prior pre-formulation factorial design to ensure that the FDCPR is not more than 20 % of the total weight. For each formulation (1-3), the FDCPR was finely triturated in a mortar and then mixed with the calculated amount of other ingredients. The granulating fluid (distilled water) was added to the mixture with minimal agitation until a thick paste of even constituency was formed before granulating using a sieve with a mesh size of 1 mm. The granules formed were dried in a laboratory oven (Model UF 75) at 42 ± 0.8 ^{o}C. The dried granules were packed in an airtight container and labeled accordingly. This procedure was repeated for all the formulations.

Table 1: Proportion of constituents of formulations by weight

Formulations	Ingredients making up the formulations (g)			
	FDCPR Extract	**Citric Acid granules**	**Tartaric Acid Pellets**	**Sodium bicarbonate powder**
1	1.20	1.13	1.89	3.28
2	1.20	1.01	1.76	3.53
3	1.20	0.95	1.58	3.78
4	1.20	0.00	0.00	0.00

FDCPR = Freeze-dried extract of *Cissus populnea* root

Determination of bulk and tapped densities

Twenty-five grams of each formulation (1-4) were separately transferred into a 25 mL measuring cylinder at an angle of 45^{o}. The height at which the granules reached (mL) was recorded and the bulk density was calculated as the ratio of the weight to the volume of the granules (g/mL) in the cylinder. The tapped density for each sample was then determined by applying one hundred taps to the cylinder containing the bulk weight of the granules and calculated as the ratio of the weight to the tapped volume of the granules (g/mL). The determinations were made in triplicates.[9]

Determination of hausner's ratio

The Hausner's ratio was separately determined as the ratio of the tapped density to the bulk density for the different formulations. The determinations were made in triplicates.[9]

$$\text{Hausner's ratio} = \frac{\text{Tapped Density}}{\text{Bulk Density}}$$

Determination of carr's index

Carr's Index was calculated from the results obtained from the bulk and tapped densities of the formulations. The determinations were made in triplicates.

$$\text{Carr's Index (\%)} = \frac{\text{Tapped Density} - \text{Bulk Density}}{\text{Tapped Density}} \times 100$$

Determination of angle of repose

The angle of repose was determined using an open-ended cylinder of fixed diameter which was placed on a base with similar diameter. Twenty grams (20 g) of each of the formulations were separately weighed and allowed to flow freely through the orifice of the funnel at an angle of 45^{o}, to form a heap whose height and diameter were determined. The determination was made in triplicate. The angle of repose was calculated using the equation below.

$$\text{Tan } \Theta = 2h/d$$

Where; h - height of the powder, r - radius of circular heap, d - diameter

Collection of blood samples

Fresh human blood sample (4 mL) from a confirmed sickle cell anemia patient who was in a steady-state and attending the routine clinic, was collected via venipuncture into heparinized bottles (EDTA bottles) at the Hematology department of the University of Ibadan - University College Hospital (UCH), Ibadan.

Preparation of red blood cells

The collected blood samples were prepared following a modified method 10. The collected human blood sample was poured separately into clean dried centrifuge tubes using needle and syringes, then centrifuged at 3000 rpm for 15 minutes. The supernatant was carefully removed with the aid of a Pasteur pipette, and the residue (packed erythrocyte) was re-suspended in freshly prepared phosphate-buffered saline (PBS), mixed carefully and gently to prevent lysis of the red blood cells. The process of centrifugation was then repeated thrice until a clear supernatant was obtained by washing the packed RBC with 1 mL PBS.

Antisickling activity evaluation

The evaluation of the granule formulations of ethanol extracts of *Cissus populnea* for antisickling activities were carried out using a modified method already described.[11,12] The resulting packed erythrocytes were washed 3 times with 0.1 mL freshly prepared *para*-hydrobenzoic acid per 1 mL of blood. The samples were then centrifuged each time for 5 min at a speed of 2000 revolutions per minute to remove the supernatant. Then 0.1 mL of the washed sickle-cell shaped erythrocytes were mixed each with 0.1 mL of the four (4) different extract formulations in uncovered test tubes and mixed with the aid of a vortex mixer. Thereafter, the mixtures were incubated at 37 ^{o}C for 3 h with occasional shaking.

Inhibitory antisickling assay testing

After incubation, 0.1 mL of 2% sodium metabisulphite was added to deoxygenate the system, then mixed thoroughly and sealed with liquid paraffin (about two drops). Samples were then taken appropriately from the four (4) formulations at 0, 45, 90 and 135 min after which the systems were incubated again at 37 ^{o}C. Each sample from the four formulations taken at intervals was smeared on a microscopic slide, fixed with 95 % methanol, dried and stained with Giemsa stain. Each sample was later examined under the oil immersion light microscope (using glycerin) and counting between 450-500 red blood cells in each sample from multiple, different fields of view across the slide using 100 microscopic magnification (40 x). The total number of both sickled and unsickled red blood cells was counted and the percentage of unsickled cells was estimated. Two types of controls were employed in this biological testing. Para-hydroxybenzoic acid (5 mg/mL) and phosphate-buffered saline (PBS) were used as positive and negative controls respectively. Blood samples collected from HbSS patients (aged between 23-30 years) were used for testing each set of experiments. The percentage mean sickling, as well as the percentage inhibition activity for each formulation, were estimated using the formulae below:

Percentage Mean sickling = Mean sickled cells x 100-Mean Total Cells

Percentage Inhibition Activity =, Control?Percentage Mean Sickled x 100/Control.

Reversal antisickling assay test

In evaluating the sickling reversal activity of crude *C. populnea* granules, 0.1 mL of the washed erythrocytes was equally mixed with freshly prepared 2 % sodium metabisulphite (0.1 mL) in a clean test tube and incubated at 37 ^{o}C for 30 min

and observed under the microscope.[13] Equal volume of the crude extracts was added to the blood-metabisulphite mixture and incubated at 37 ^{o}C for another 30 min. Thereafter, samples were taken and observed at 30 min interval from 0 min up to 90 min. The smear preparation, counting of sickled and unsickled cells and microscopic images of depranocytes/sickle cells were examined.[12] A drop of the sample containing each formulated recipe was smeared on a microscope slide and viewed under a medium-powered magnification (x 10) under the oil immersion. Cells were counted on multiple fields of view on each slide after which the mean cells were calculated. The number of sickled and unsickled cells was then counted to determine the total number of cells.[10,11] The percentage mean sickling, as well as the percentage reversal activity for each extract, was estimated using the formulae below:

Percentage Reversal Activity = Contro–Percentage Mean Sickled x 100/Control.

The total number of cells counted = No of sickled + No of unsickled cells.

Cells were counted as being also performed for inhibitory activity.

RESULTS

The constituents of the ingredients that make up the formulations are presented in Table 1, with the freeze-dried extract of *Cissus populnea* root (FDCPR) incorporated as the principal (active) ingredient. Citric acid, tartaric acid and sodium bicarbonate, which are usual components of effervescent formulations, were included in different concentrations. Formulation 4 contained only the FDCPR. The flow and density measurements of the four (4) formulations are shown in Table 2, and the results showed that the bulk and tapped densities of the formulations followed a similar pattern in ranking (1>3>2>4) while it was observed that the Hauner's ratio (HR) and Carr's Index (CI) had a reverse relationship, with the crude extract granules having the highest HR (1.03±0.53) and lowest CI (18.32±0.03) values. Angle of repose ranked formulations 4>3>2>1. Plots of the percentage of unsickled cell versus time for reversal and inhibitory antisickling activities of the formulations (along with phosphate buffered Saline (PBS) and para hydroxyl benzoic acid (PHBA) are shown in Figure 1 and 2 respectively. Formulations 3 and 4 showed the highest antisickling (72.0 % and 68.0%) and reversal activities (57.0 % and 59.0 %) comparable to PHBA (64 % inhibitory and 56.0 % reversal activities).

Table 2: Flow and Density measurements of effervescent granules (mean ± SD, n=3)

Parameters	Formulations			
	1	2	3	4
Bulk Density (g/cm^3)	1.37±0.03	1.13±0.11	1.18±0.13	1.07±0.07
Tapped Density (g/cm^3)	1.68±0.02	1.51±0.07	1.69±0.03	1.31±0.13
Carr's Index	18.45±0.11	25.17±0.08	30.18±0.12	18.32±0.03
Hauner's ratio	0.82±0.03	0.75±0.13	0.70±0.08	1.03±0.53
Angle of repose (^{o})	29.13±0.17	31.03±0.27	34.18±0.28	39.18±0.11

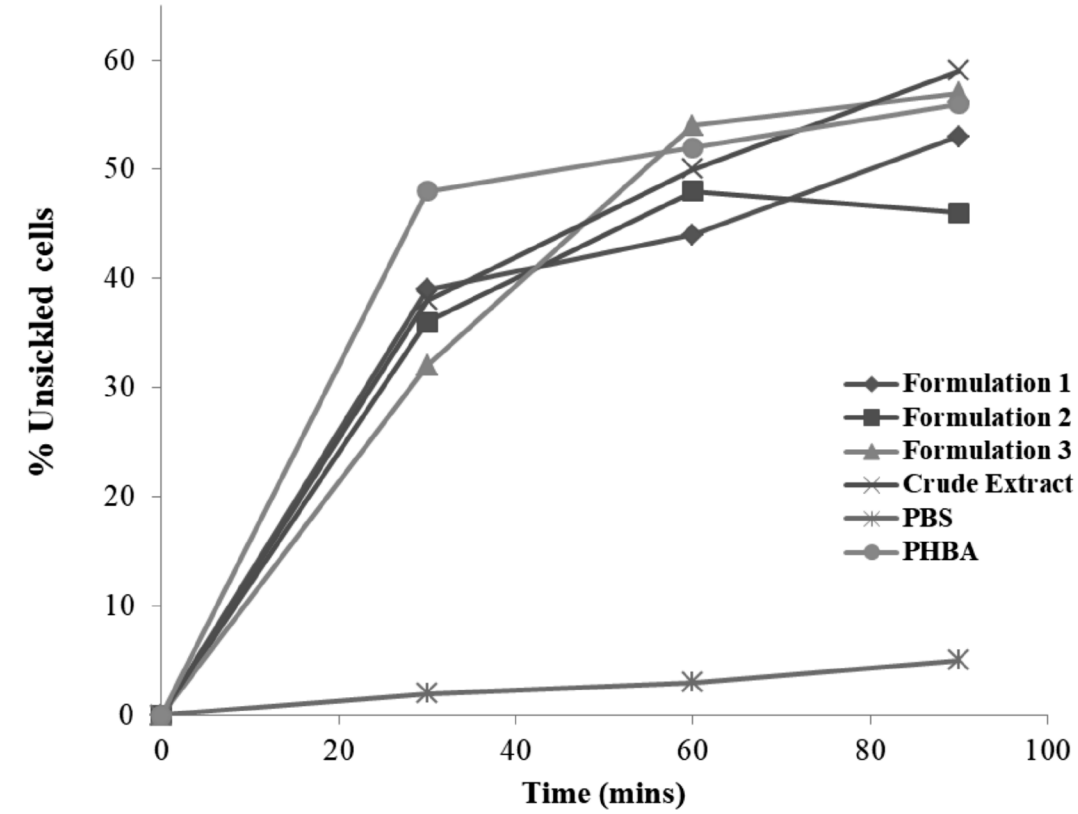

Figure 1: Plot of percentage unsickled cells against time (min) for the reversal antisickling activities of different formulations (PBS = Phosphate Buffered Saline, PHBA = Para Hydroxyl Benzoic Acid

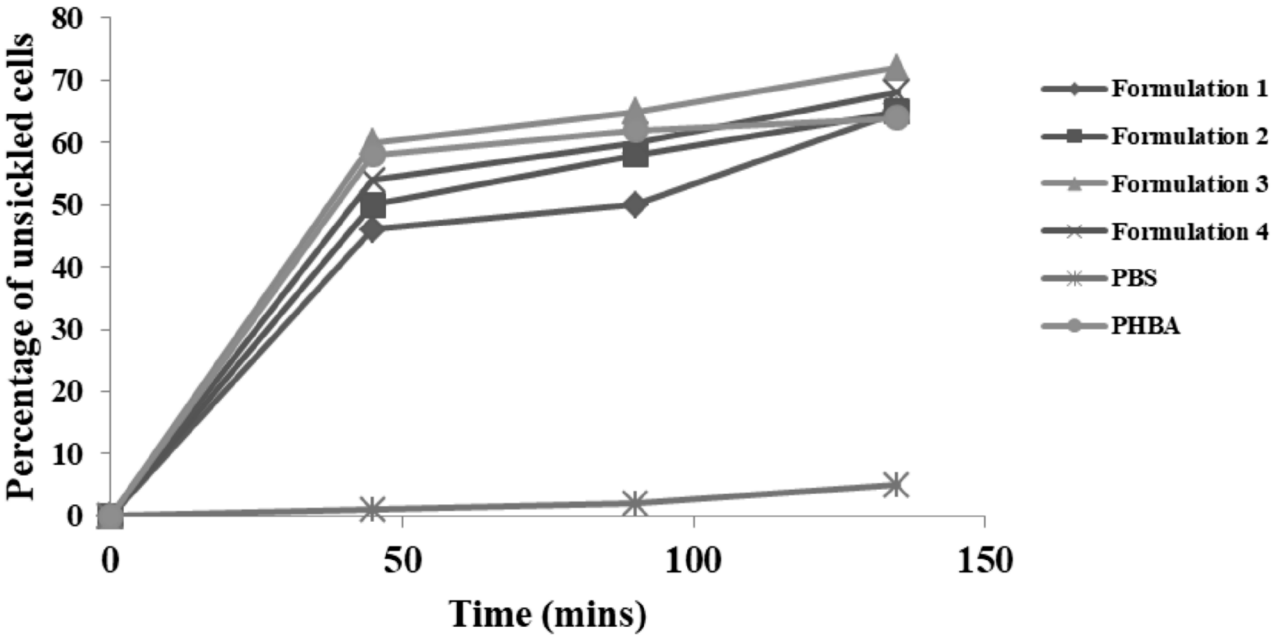

Figure 2: Plot of percentage unsickled cells against time (min) for the inhibitory antisickling activities of different formulations. PBS = Phosphate Buffered Saline, PHBA = Para Hydroxyl Benzoic Acid

DISCUSSION

The uniqueness of effervescent granules lies in their ability to release gases when dissolved in water, providing a soothing effect for the patient while also enhancing the absorption of the active ingredient due to the presence of citric and tartaric acids.[14] The quality of granules is closely linked to their flow properties, which are crucial in determining the final product's weight, content uniformity, and physical consistency in shape and size.[15] Various parameters, including bulk and tapped densities, Carr's Index, Hausner's ratio, and angle of repose, are commonly used to assess flow properties and are well-established pharmacopoeia standards.[16]

Bulk density provides insight into the tendency of powders to adhere to each other before external pressure is applied.[16] It also helps predict the ratio between smaller and larger particles in a granule bed, ultimately influencing packing behaviour.[17] In this study, Formulation 4 (pure extract of *Cissus populnea*) exhibited the lowest bulk and tapped density values, suggesting that the presence of citric acid, tartaric acid, and sodium bicarbonate in other formulations contributed to denser granules. The ranking of bulk and tapped densities was Formulation 1 > 3 > 2 > 4 and Formulation 3 > 1 > 2 > 4, respectively.[18]

Sodium bicarbonate has the ability to confer sphericity to formulations in which it is added as an excipient,[18] and the results obtained from Hausner's ratio, which measures densification during handling and is proportional to resistance to flow, ranked the formulations as 4 > 1 > 2 > 3, indicating that Formulation 3 (with the highest sodium bicarbonate content) had the best flow properties. The Carr's Index values followed a similar trend (3 > 2 > 1 > 4), confirming that sodium bicarbonate contributed to improved flow. The angle of repose, an indirect indicator of granule flowability, was below 40 ^{o}C for all formulations, signifying good flow properties.[19] Formulation 1, which contained the highest proportion of tartaric acid, exhibited the lowest angle of repose, suggesting superior flow behaviour.

The reversal antisickling activity of the formulations was evaluated over time. At 0 minutes, none of the formulations exhibited activity, as they had not yet interacted with the sickled red blood cells. However, after 30 minutes, the reversal activities were: Formulation 1: 39 %, Formulation 2: 36 %, Formulation 3: 32 %. Formulation 4 (pure extract): 38 %, PBS (negative control): 2 %, PHBA (positive control): 48 %. After 60 minutes, the reversal activities increased as follows: Formulation 1: 44 %, Formulation 2: 48 %, Formulation 3: 54 %, Formulation 4: 50 %, PHBA: 48 %, PBS: 2 %. At 90 minutes, the rankings were: Formulation 4 (pure extract): 59 % (highest), Formulation 3: 57 %, PHBA: 56%, Formulation 1: 53 %, Formulation 2: 46 %, PBS: 5 % 20.

These findings from this study show that Formulation 4 (pure extract) had the highest reversal antisickling activity comparable to PHBA. Formulation 3, which contained the highest sodium bicarbonate concentration possessed moderate reversal activity likely due to sodium bicarbonate's role in improving local vascular blood flow and correcting acidosis-a known trigger of sickling.[21]

At 0 minutes, none of the formulations exhibited inhibitory antisickling activity. However, after 45 minutes, the inhibitory effects were: Formulation 1: 46 %, Formulation 2: 50 %, Formulation 3: 60 %, Formulation 4 (pure extract): 54 %, PHBA (positive control): 58%, PBS (negative control): 1 %. At 90 minutes: Formulation 3: 65% (highest), PHBA: 62 %, Formulation 4: 54 %, Formulation 2: 50 %, Formulation 1: 48 %, PBS: least effective.

After 135 minutes, Formulation 3 maintained the highest inhibitory effect (72 %), while Formulations 2 and 4 exhibited higher inhibitory antisckling activity than PHBA. These results suggest that sodium bicarbonate significantly enhanced the inhibitory activity, likely by maintaining an alkaline environment that prevents erythrocyte sickling .[22]

Although all formulations contained the same quantity of *Cissus populnea* extract (1.20 g), their effectiveness varied due to differences in the effervescent excipient compositions. Specifically, sodium bicarbonate contributed to both inhibitory and reversal activities by improving blood flow and buffering against acidosis, a known promoter of sickling.[21]

CONCLUSION

The results of this study suggest that effervescent granules of *Cissus populnea* exhibit significant antisickling activity. Formulation 4 (pure extract) demonstrated the highest reversal antisickling activity, while Formulation 3 (with the highest sodium bicarbonate content) showed the greatest inhibitory effect. The rapid dissolution of the effervescent granules (within 2 minutes) offers a potential advantage for pediatric and adult patients with sickle cell disease, potentially improving compliance and

therapeutic outcomes. However, further studies are needed to determine the effect of granules containing different concentrations of the *Cissus populnea* extract and effervescent excipients for maximum antisickling activity. Extensive toxicity, pharmacokinetic profiling can also be carried out to establish the safety in long term use of the granules. By addressing these areas, the therapeutic potential of *Cissus populnea* in sickle cell anemia management can be better understood and optimized.

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The authors declare no conflict of interest.

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