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Wound healing and immunomodulatory effects of juice from *Bidens pilosa* fresh leaves

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Abstract
In response to a wound, a physiological restoration phenomenon occurs wound healing. This phenomenon can evade the body's control and lead to a wide range of disability. A major drawback of modern wound-healing drugs is their accessibility. To overcome this problem, people turn to medicinal plants. This study aimed to evaluate the *in vivo* healing and immunomodulatory activities of *Bidens pilosa* leaf juice. This work began with harvesting fresh *Bidens pilosa* leaves in the western region and their identification at the Herbarium, followed by mechanical pressure extraction to obtain *Bidens pilosa* Juice. Phytochemical screening was carried out on the extract obtained. Acute oral and cutaneous toxicity tests were carried out in accordance with OECD lines 425 and 402. Healing activity was assessed on a non-infected wound model generated by skin excision of a 2x2cm area. Immunomodulatory activity was assessed by two tests: carbon clearance with determination of the phagocytic index, and cyclophosphamide-induced neutropenia with leukocyte and platelet counting on 3 occasions. The yield of *Bidens pilosa* juice extraction was 60%. Phytochemical screening revealed the presence of flavonoids, polyphenols, tannins and saponins. The wound-healing activity showed that all doses of *Bidens pilosa* juice tested increased the speed of wound healing, compared with BIAFINE* (trolamine 0.0670g/100g), a pharmacy-available wound-healing agent. *Bidens pilosa* juice at all tested doses decreased the phagocytic index like that of cyclophosphamide, a reference immunosuppressant indicated for inflammatory wounds and an increase in leukocytes and platelets after cyclophosphamide-induced neutropenia. The results obtained confirmed that the juice of *Bidens pilosa*'s fresh leaves has wound healing and immunomodulatory activity *in vivo*, while having zero oral and cutaneous toxicity.

Keywords: *Bidens pilosa* juice, phytochemical screening, wound healing, immunomodulation

1. Introduction
The skin is the most extensive and multifunctional organ of the organism, separating the internal and external environments. The discontinuity of the skin lining due to thermal, chemical, or physical damage constitutes a wound. In response to a wound, healing is a non-physiological phenomenon that restores the integrity of the skin after accidental or intentional trauma. The healing process occurs through four stages: haemostasis, inflammation, proliferation, and remodelling. When just one of these stages does not progress as usual, it can lead to a chronic open wound [1-4].
In the inflammatory phase, mainly involving the activation of the innate immune system, neutrophils and monocytes rapidly migrate into damaged skin. Resident skin cells, for example, keratinocytes, macrophages, dendritic cells and mast cells, are exposed to danger signals and will subsequently promote the production of soluble pro-inflammatory mediators. In response, neutrophils are recruited from the circulation to the wound site in the early inflammatory stage, or they perform phagocytosis to remove pathogens and cellular debris. About 3 days after injury, monocytes are recruited to the injury site, where they differentiate into macrophages and promote healing [2-3]. Pathological figures such as diabetes mellitus, chronic conditions, and peripheral vascular diseases can lead to wounds. These wounds can be acute or chronic ulcerations depending on the healing time [4]. Wounds reduce quality of life by causing serious infections including gangrene or tetanus, immobility, or pain in a high percentage of patients and would therefore constitute a public health problem [5-6].

Very few modern drugs are used externally for the treatment of wounds like dexpanthenol and trolamine unfortunately, they are both often difficult to access in resource-limited countries. Population in developing world are using traditional herbal medicine ion first intention to manage their health issues including wound healing. *Bidens pilosa* is a traditional plant from Cameroon that belong to the Tracheobionte subkingdom, the Spermatophytes super division, Magnoliophyta Division, the Magnoliospida Class, Asterides Subclass, Asterales Order, Asteraceae Family, *Bidens* Gender and the *Bidens pilosa* L. species. The plant is commonly known as bristling bident, hairy bident, needle herbs (French). In English: blackjack (in English), ntsohoho (in Comoros), anatsinahy (in Malagasy), Gui Zhen Cao or Feng Xian Cao (in Chinese) [7], tsuetsueneck (in West region of Cameroon). *B. Pilosa* has a wide range of traditional utilisations. It is used as food by both humans and livestock. However, it is not included in the milch cow food as the essential oil contained in his leaves tends to give the worst quality milk. It is an anti-infective, uterotonic, wound healing, hypertension, and conjunctivitis and stops the bleeding after injuries. Many scientific facts have been reported in the literature review. Alkaloids, flavonoids, phenol, tannins, and steroids were identified in extracts of *Bidens pilosa* depending on the extraction solvent [8]. The effect of the aqueous leaf extract on haemostasis while the methanolic extract of the leaf has a hypotensive effect while the ethanolic extract has shown relatively weak wound healing effects [9-10]. The infusate and the essential oil from the roots and the twigs are reported to have antibacterial and antifungal effects [11–13]. Butanolic extracts of the whole plant have shown anti-inflammatory and antiallergic potential when applied to respiratory ways [14-15]. To the best of our reading, we did not find a report of the plant using the leaf juices to mimic the traditional utilisation of the plant for wound healing and how it interacts with the immune system to have that effect. Therefore, this study aimed at evaluating the wound healing and immunomodulatory effect of *Bidens pilosa* leaf juice.

2. Materials and Methods

2.1 Plant material

The leaves of *Bidens pilosa* were harvested in the western region of Cameroon, more precisely in the town of Bafoussam. The plant was subsequently identified at the National Herbarium of Cameroon (HNC) in comparison with the specimen Asteraceae *Bidens pilosa* under the number 58712/HNC.

2.2 Animal housing

The rats were of the Wistar strain of the species Rattus norvegicus. They were raised in the animal facility of the pharmacology laboratory of the Faculty of Medicine and Pharmaceutical Sciences of the University of Douala. They were fed 24 hours a day and drank drinking water ad libitum with 12h light/dark cycle.

2.3 Animal selection criteria

The rats were selected based on the following criteria: pregnant rats, rats aged less than 2 months or more than 2.5 months, and rats weighing less than 150g or more than 200g were not included in the study. Rats showing no physical and behavioural abnormalities, non-pregnant, aged between 2 and 2.5 months and weighing 150 and 200g were included in the study while the rat that became sick or had strange behaviour were excluded from the study.

This study received approval from the ethical comity (approval number 3593 CEI-Udo/05/2023/T)

2.4 Extract preparation

The juice from the fresh leaves of *Bidens pilosa* (SB) was prepared as follows: the fresh leaves were first washed and rinsed with clean running water and then weighed. They were then ground with distilled water at a rate of 10ml/100g in a blender. Finally, the porridge obtained was filtered on Wattman n°3 filter paper to extract the juice. The juice was stored in a dark bottle and placed in a cooler containing Cryopacks and broth in the lab and freeze-dried to prolong the preservation.

The yield of extraction was evaluated using the following formula: Yld = (wj/wl) x100 where *wj* is the weight of the juice obtained and *wl* is the weight of fresh leaves.

2.5 Phytochemical screening of the juice from fresh leaves of *Bidens pilosa*

The phytochemical screening was carried out to identify the main classes of compounds present in the crude juice (called juice here). Two principals were considered: precipitation by the formation of insoluble complexes and colourimetry by the formation of soluble coloured complexes. The tests were as follows [16, 8]:

2.5.1 Liebermann-Burchard test

Purpose: to identify sterols and terpenes

Procedure:

- Dilute 1 ml of crude juice in 1ml of chloroform.
- Add a few drops of acetic anhydride and concentrated sulfuric acid sequentially.

2.6 Results and interpretation

The appearance of a blue colour that turns to dark green shows the presence of sterols and the appearance of a brick red colour turns to purple for terpenes. The control is carried out with the betulinic acid solution.

2.6.1 Shinoda test

Purpose: Identify flavonoids

2.6.2 Procedure

- Shake for 5 to 10 minutes 0.2 g of the crude juice in 10ml of methanol
- Add 1 ml of concentrated HCl, 0.2 g of magnesium shavings and a few drops of concentrated sulfuric acid sequentially after shaking each time.

2.7 Results and interpretation

In the presence of flavonoids, a red-orange colour is obtained for the flavones, a cherry red colour for the flavanols and a purplish red colour for the flavanones.

2.7.1 Dragendorff test

Purpose: To identify the alkaloids

2.7.2 Procedure

- Dragendorff's reagent was prepared by mixing an equal volume of solution A (solution containing 0.85 g of bismuth nitrate dissolved in 10ml of acetic acid and 40 ml of distilled water) and a solution B (solution containing 8 g of potassium iodide in 20 ml of distilled water).
- Dissolve to 1ml of crude juice, few drops of Dragendorff's reagent were added and gently shake.

2.8 Results and interpretation
The presence of alkaloids is materialized by the presence of a yellow-orange precipitate.

2.8.1 Foam index test
Purpose: Identify saponins

2.8.2 Procedure: Dilute 1ml of the crude juice in 4ml of distilled water then shake energetically for 15 seconds then let stand for 15 min.

2.9 Results and interpretation
The appearance of foam at a height of 1cm that persists for at least 15min indicates the presence of saponin.

Bate Smith test
Purpose: Identify tannins
Procedure: Dilute 1 ml of the crude juice in 10 ml of distilled water and add 2 ml of FeCl3 to 2 ml of the previous solution.

2.10 Results and interpretation
Obtaining a brown-black precipitate indicates the presence of tannins.

$FeCl_3$ test
Purpose: To identify phenolic compounds
Procedure: Add 1 to 3 drops of alcoholic solution of 2% ferric chloride in 2 ml of juice and gently shake.

2.11 Result and interpretation
The appearance of a dark-blue or dark-green colour. The control is carried out with the gallic acid solution.

2.11.1 Bornstrager test
Purpose: Identification of anthraquinones
Procedure: Add 1ml of ammonia only to 1ml of juice
Result and Interpretation: the appearance of a purplish-red colour reflects the presence of anthraquinones.

2.12 Toxicity assessment
2.12.1 Acute oral toxicity
Dose adjustment method OECD 425, 2022 [17].
The principle is based on the use of cohorts of animals of the same sex exposed orally to maximum doses of products to be tested in these case doses of 2000 mg/kg of body weight. Briefly, animals were divided into two groups of three animals each. The first group was our control and received orally distilled water. The second group was our test group and receive the Juice (reconstituted from lyophilized) at the dose of 2000 mg/ml orally. All the groups were then observed for 30min, 1h, 2h, 4h, 12h, and 24h after administration then every 2 days until the day 14th. The parameters observed were Stool appearance, coat appearance, eye colour, mobility, aggressiveness, mortality, tremor, convulsion, urine appearance, and mass variation.

2.12.2 Acute Dermal Toxicity
Predetermined Dose Method OECD 402, 2017 [18].
The principle is based on the use of cohorts of animals of the same sex exposed dermally to doses of products to be tested in these case doses of 2000 mg/kg of body weight. Briefly, animals were divided into two groups of three animals each. Before the application of the drugs, all the animals were shaved carefully to avoid lesions on their skin on the dorsal flank. The treatments were the apply to each animal according to the batches then the contact with the skin was maintained for 24 hours by applying a compress and adhesive plaster. All the groups were then observed for 30min, 1h, 2h, 4h, 12h, and 24h after administration then every 2 days until the day 14th. The parameters observed were Stool appearance, coat appearance, eye colour, mobility, aggressiveness, mortality, tremor, convulsion, urine appearance, and mass variation.

2.13 Evaluation of the healing activity: Methods of wounds by excisions
This test is based on the monitoring of the excision-induced wound constriction in the presence of the wound-healing drug using the protocol described by Namunana *et al.*, [19] with slight modifications. Briefly, animals were divided into 4 groups of 5 animals each. After being weighed, animals were anaesthetised using a combination of 2ml of ketamine chloridrate (50 mg/ml) with 1 ml of diazepam 5mg/kg by intraperitoneal route. After a few minutes, animals were shaved and a piece of 2x2 cm of shaved skin was cut off using forceps and scissors. The treatment was applied to the animal group in batch as follow:

Batch 1: Negative control the five rats of the batch received distilled water.
Batch 2: Positive control. The 5 rats of this batch received Trolamine.
Batch 3: The 5 animals of the batch received the juice dermally at the dose of 5 mg/ kg body weight.
Batch 4: The 5 animals of the batch received the juice dermally at the dose of 50 mg/kg body weight.

Macroscopic appearance with a description of the wound (redness, swelling, exudate, hard crust, bleeding, and suppuration) and calculation of the contraction rate of the wound according to the following formula:

$$Cr= ((S0-Sn)/S0)*100$$

Where Sn is the surface of the wound on day n and S0 is the surface of the wound on the induction day.

2.14 Evaluation of immunomodulatory activity
2.14.1 Carbon clearance method
The carbon clearance test assesses the effect of drugs and phytoconstituents on the reticuloendothelial system (RES). The RES comprises a diffuse system composed of phagocytic cells. Once the colloidal carbon particles are directly injected into the blood, they are removed by RES through phagocytosis. Rapid removal of carbon particles has been associated with increased phagocytic activity. The protocol used was as previously described by Ganeshpurkar and saluja [20] with modifications brought by Bafna *et al.*, [21]. Briefly, animals were clustered into 5 groups of five animals each as followed.

Batch 1: This batch was normal, not receiving any substance nor included in any gavage.
Batch 2: This was negative control, receiving distilled water orally by gavage each day.
Batch 3: This was our positive control, receiving Cyclophosphamide 30mg/kg body weight/day
Batch 4: This batch received the juice at the dose of 5mg/ kg body weight/day.
Batch 5: This batch received the juice at the dose of 50mg/ kg body weight/ day.

On day 6, all animals involved in the study received

intraperitoneally, 0.1 ml / 100 g body weight of an India ink suspension and the bool were subsequently collected via the tail vein in dry tubes at times 0 min and 15 minutes. To 50µl of collected blood, 4 ml of sodium carbonate (1% in sterile distilled water) was added and the absorbances were recorded at 660 nm.

The carbon clearance rate was calculated using the following formula:

Ccl= (LnOD1-LnOD2)/ (t2-t1) where OD1 is the optical density at time 1 and OD2 is the optical density at time 2. T1 is 0 minutes after injection and t2 is 15 minutes after injection.

2.14.2 Cyclophosphamide-induced neutropenia method

Cyclophosphamide is an anticancer agent that induces leukopenia. Prevention of cyclophosphamide-induced immunosuppression is another measure of immunomodulatory effect. In this report, the method used was as described by Ganeshpurkar and saluja [20] with slight modifications. Briefly, 20 animals were clustered into four batches of 5 animals each as follows.

Batch 1: This batch was normal, not receiving any substance nor included in any gavage.

Batch 2: This was negative control, receiving distilled water orally by gavage each day.

Batch 3: This batch received the juice at the dose of 5 mg/ kg body weight/day.

Batch 4: This batch received the juice at the dose of 50 mg/ kg body weight/ day.

Before treatment, the blood was collected from all animals by retro-orbital vein in EDTA tubes to constitute the baseline. Then, all animal received their drugs or water by oral route daily for 10 days. And on day 10th, the blood was again collected under the same conditions and a neutropenic dose of cyclophosphamide (200 mg/kg of body weight) was administered by intraperitoneal route to all animals. The blood was once more collected in the same condition 48 hours later. The entire sample was submitted to the counter coulter to count the number of leukocyte and pellet as they are playing a major role in wound healing effect.

2.15 Statistical analyses

The data were statistically analysed using STATGRAPHIC SIGMA for Windows for the analysis of variance at the significance threshold of 0.05. Microsoft Excel 2019 for Windows help to calculate means, and standard deviations, and draw graphs.

3. Results and discussion

3.1 Results

3.1.1 Mechanical extraction of juice from *Bidens pilosa* leaves

From 750 g of fresh leaves 450 g of juice were obtained given a yield of 60%. After lyophilisation, 27.5 g of powder was obtained giving a yield of 3.67 g of organic extractable per 100g of wet leaves.

Phytochemical screening

Table 1: The result of the phytochemical screening

Test	Compounds group	Observation	Conclusion
Lieberman Bouchard	Sterol and triterpenes	Green blue or red-purple colour	-
Shinoda	Flavonoids	Orange-red colour	+
Dragendorff	Alkaloids	Red orange precipitate	-
Foam indices	Saponins	Persistent foam	+
Bate smith	Tannins	Dark brown precipitate	+
$FeCl_3$	Phenolic compounds	Dark blue colour	+
Bornstager	anthraquinones	Red purple colouration	-

+ = presence and - = absence

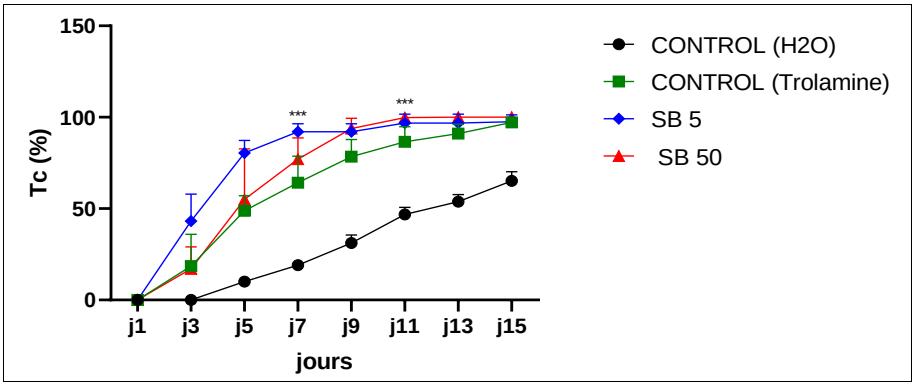
From table 1, it comes out that, terpenoids, alkaloids and anthraquinone were absent from our juice.

3.1.2 Acute oral and dermal toxicity

No signs of toxicity were observed in both tests suggesting that the juice from *Bidens pilosa* is safe to use at up to 2000 mg/kg body weight in rats.

3.1.3 Wound healing activity

The wound contraction was shown to be proportional to the concentration of our juice over time (Fig 1). Juice at the concentration of 5 mg/Kg BW tends to reduce the length of the wound faster than that at 50 mg/Kg BW and Trolamine from day 3 to day 7. But, from day 9 to the end of the experiment, all the doses of the Juice and trolamine totally heal the wound.



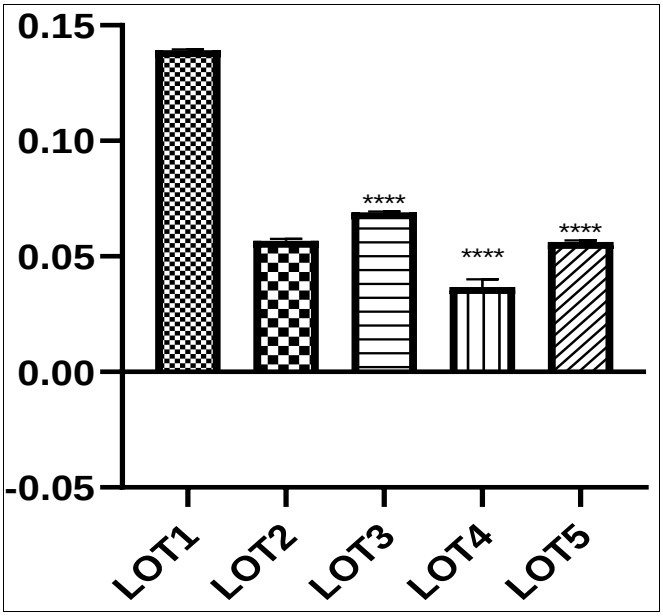
SB5= *Bidens pilosa* juice at 5 mg/kg BW: SB50 = *Bidens pilosa* juice at 50 mg/kg BW; TC = contraction rate; J= day

Fig 1: Contraction percentage over 15 days

3.1.4 Immunomodulatory effect

3.1.4.1 Carbon clearance tests

The results of the phagocytic index after a 5-day treatment of the different batches are summarised in Fig 2. This highlight that the phagocytic index of the batches treated with juice decreased significantly (p>0.05) compared to the batches to the other batches. The highest phagocytosis index was obtained with the SB 50 extract.



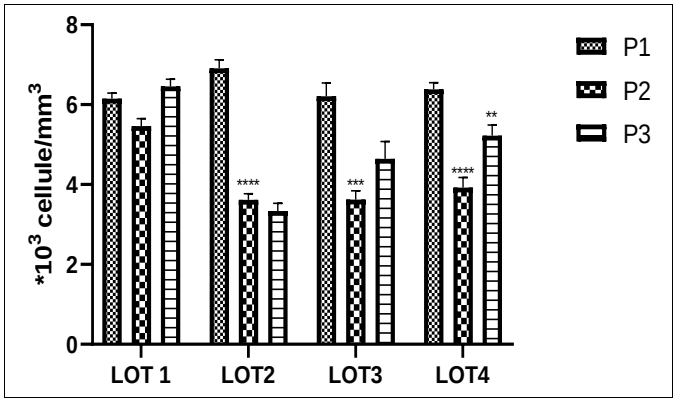
LOT1= batch constituted of normal animals, LOT2= batch of animals that received distilled water, LOT3= bach of animals that receive cyclophosphamide, LOT4= batch of animals that receive *Bidens pilosa* leaf juice at 5 mg/Kg BW, LOT5= Batch of animal that receive *Bidens pilosa* leaf juice at 50 mg/Kg BW.

Fig 2: Carbon clearance in function of different treatments

3.1.4.2 Cyclophosphamide-induced neutropenia

During this test, the blood count of the different batches was carried out after three samples were taken respectively on day 0 and day 10 of treatment and 48 hours after induction of neutropenia by cyclophosphamide. The parameters of the complete blood count that we used were the number of leukocytes and thrombocytes involved in the healing process.

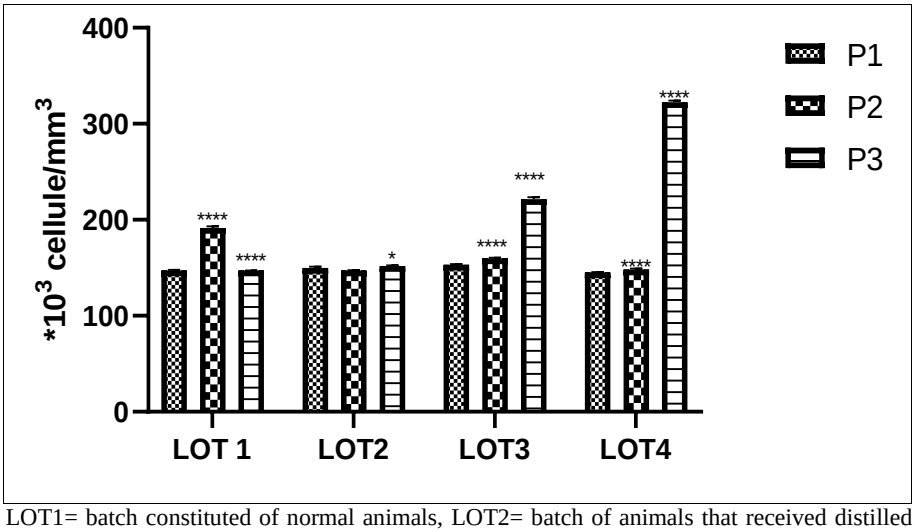
The number of leukocytes dropped drastically after 10 days of treatment in the test batches compared to the normal batch. This number then increased 48 hours after the administration of cyclophosphamide, which took place on day 10. The greatest increase in leukocytes was obtained with the SB 50 extract (Fig 3).



LOT1= batch constituted of normal animals, LOT2= batch of animals that received distilled water, LOT3= batch of animals that receive *Bidens pilosa* leaf juice at 5 mg/Kg BW, LOT4= Batch of animal that receive *Bidens pilosa* leaf juice at 50 mg/Kg BW, P1: sample on day zero, P2: sample on day 10, P3: sample 48 hours after cyclophosphamide

Fig 3: Leucocyte number at different collection times

As for the platelets, they did not change significantly during the 10 days of treatment in the test batches compared to the normal batch, but after the induction of neutropenia the number of platelets was seen to be significantly (p>0.05) increased in treated batches relative to other batches. The greatest platelet increase was obtained with the SB 50 extract (Fig 4).



LOT1= batch constituted of normal animals, LOT2= batch of animals that received distilled water, LOT3= batch of animals that receive *Bidens pilosa* leaf juice at 5 mg/Kg BW, LOT4= Batch of animal that receive *Bidens pilosa* leaf juice at 50 mg/Kg BW, P1: sample on day zero, P2: sample on day 10, P3: sample 48 hours after cyclophosphamide.

Fig 4: Evolution of pellet numbers

4. Discussion

The present work focuses on analysing the juice obtained by mechanical extraction of the fresh leaves of *Bidens pilosa* for their wound healing and immunodulatory effect. The results show that the extraction yield was 60%, of a greenish liquid having water content greater than 93.8%. This is the first time

this type of preparation if reported. This extraction strategy was adopted based on the traditional utilisation of the plant for wound management purposes in different region of Cameroon. The phytochemical study showed that the juice contains polyphenols, saponins, flavonoids and tannins. This aligns with reports that reported the presence of saponins, flavonoids phenolic compounds and tannins in the aqueous extract of dried leaves of *Bidens pilosa* [7, 8, 12, 19]. The results obtained during the evaluation of the acute oral and cutaneous toxicity of *B. pilosa* juice after a single administration of 2000 mg/kg according to the OECD did not reveal any signs of toxicity between the batches treated with juice and those treated with distilled water. No change in the behavior of the rats, no deaths and no significant changes in the weight mass of the rats between the different batches were reported. These results are in agreement with those reported previously. From the above it appears that the samples tested are considered non-toxic at doses below 2000 mg/kg, according to the OECD limit test at 2000 mg/kg body weight [17, 18]. this is constant with the folkloric utilisation of the plant as it is an edible plant that have never show any harmful effect to the consumers [7, 13-15, 22, 24, 25]. Traditionally *Bidens pilosa* is used in the management of wounds [7, 14, 15]. This was confirmed through this study as the juice of the fresh leaves significantly increased the wound contraction rate compared to the untreated (negative control). The wound contraction was proportional to the concentration of the juice. To the best of our reading, we did not find any researches that have been made on juice of *Bidens pilosa* leaves. However, these results go in the same line as that from Hassan *et al.*, [25] who obtained a healing potential with the ethanolic extract of the dried leaves of *Bidens pilosa* collected in Kampala. Furthermore, this results in view of the phytochemical screening, comfort the presence of wound healing compounds such as flavonoids [26] and various type of polyphenol [27, 28] that are known to play a key role in healing stage name inflammation and immunomodulation [9, 26, 27, 29, 30]. The carbon clearance test is a test that has been performed to assess the effect of substances on phagocytic activity [20, 21]. In the present study, the juice of *Bidens pilosa* reduced the phagocytic activity of macrophages. At the highest dose (50 mg/kg body weight) the juice was comparable to immunosuppressant, cyclophosphamide, used here as positive control. However, at the lowest dose tested (5 mg/kg body weigh) the phagocytic capacity was more important than that of negative control. Taken together, the phagocytic effect seems to be dose dependant. In fact, at lower doses, the juice is immunostimulatory and at the higher dose the juice is immunosuppressor. This contrast goes in the same line with reports in the literature. First, Chiang *et al*., has observed that ethyl acetate isolated from *Bidens pilosa* decrease the production of nitric oxide produce by macrophages and thereby reduce the phagocytic index [31]. In another hand, Chang *et al.*, reported that crude extract increased interferon gamma (IFN-γ) promoter activity. This INF is an important cytokine that play role in immune response and phagocytic effect [32]. The cyclophosphamide-induced neutropenia test was performed in three phases. The first phase was done on day zero with a blood sample to count leukocytes and platelets that are known to be miles stone in the wound healing process [1, 2]. The second phase of our assay consisted of counting of leukocytes 10 days post treatment. From these, a significant decrease (p>0.05) in leukocytes in treated animal in compared to the normal control was observed. To the best of our reading, we did not find any report on the effect of the leaves juice of *Bidens pilosa*. However, Bleyer *et al*., working on aqueous extract had reported that their extract caused neutropenia and monocytopenia *in vitro* on human blood [33]. Moreover, after induction of immunosuppression with cyclophosphamide, the level of Leucocyte jumps up significantly in comparison to the blood from untreated animal. These observations can be explained by the parallel report on the same plant. The production of cytokine observed by Chang *et al*., [32] is one of the fact that stand for the observed effect. The variation in the platelets count was significantly different from the treated at the dose of 50 mg/kg body weight in comparison to the control 10 days treatment. This goes along the line with the report by Pade *et al*., [10].

The present study suggests highlight the wound healing and the immunomodulatory effects of the juice from the fresh leaves of *Bidens pilosa*. Thereby confirming the traditional safe usage of *Bidens pilosa* for wound management. The present study spot a light on the formulation of a standardised wound healing drug that could be used as cataplasm by population from least income countries.

5. Declaration of Conflicting Interests

Authors declared no conflict of interest.

6. Funding acknowledgement

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