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QUALITATIVE PHYTOCHEMICAL STUDY AND EVALUATION OF THE HEALING ACTIVITY OF THE COMBINED CRUDE AQUEOUS EXTRACTS OF TWO ASTERACEAE : CHROMOLAENA ODORATA AND THITONIA DIVERSIFOLIA

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ABSTRACT

The practice of traditional medicine constitutes an important heritage for the African continent. The active ingredients of medicinal plants are used to overcome several pathologies, in particular wounds. The wound corresponds to an interruption of the skin covering. The biological material necessary for this study consisted of plant material, consisting of the leaves of *Chromolaena odorata* and *Tithonia diversifolia* and animal material consisting of white wistar rats. The extraction yield was calculated by calculating the ratio of the mass of the extract obtained to the mass of macerated powder. Phytochemical screening was carried out using analytical techniques. The acute toxicity experiment was conducted according to guideline 423 of the OECD protocol. The wound induction technique by excision was used. The respective extraction yields of *Chromolaena odorata* and *Tithonia diversifolia* were 6.2% and 1.5%. The tests for alkaloids, anthocyanins, anthraquinones, flavonoids, polyphenols, saponins, steroids, catechin tannins, triterpenes, phenols were positive with the aqueous extracts of the two species except phenols for *Chromolaena odorata* and catechin tannins for *Tithonia diversifolia.* The acute toxicity study revealed an LD50 greater than 5000 mg/kg. Four clinical wound parameters were observed during this study. The study of wound healing induced by skin excision was conducted over a period of 18 days. The wounds in the

negative control group took the longest (18 days) to heal. Only the combination of extracts administered at a dose of 200 mg/kg was able to heal the wounds in a period of 12 days. The extracts were therefore found to be effective for the treatment of wounds and good for the formulation of healing ointments.

INTRODUCTION

The practice of traditional medicine is an important heritage for the African continent (Dibong *et al.*, 2018). The active ingredients of medicinal plants are used to overcome several pathologies including wounds. Research into drugs that can accelerate wound healing is a developing branch of modern biomedical science (Okwu, 2004). Traditional medicine intervenes in the treatment of certain diseases due to the combination of the use of medicinal plants and scientific research. Medicinal plants are increasingly used to the detriment of the use of modern drugs such as antibiotics whose germs have developed resistance in several infections. Therefore, herbal preparations may prove more effective than those of modern medicine and could be administered over a longer period (Okwu, 2005). Research has been carried out to scientifically evaluate the healing activity of plants whether with monospecific recipes or combined recipes (Rabia, 2016). The wound corresponds to an interruption of the skin covering. It can be superficial, involving only the epidermis, part of the dermis or be deep with exposure of the subcutaneous tissue. Its evolution depends on its extent and depth but also on local or general factors that can slow down or prevent its healing. This work aims to study the healing properties of the combination of aqueous extracts of *Chromolaena odorata* and *Tithonia diversifolia* through the determination of extraction yields, the highlighting of large groups of secondary metabolites, the evaluation of the acute oral toxicity of the combined aqueous extracts, the test of the healing activity of the combined aqueous extracts of the two species, compared with that of Biafine on rats whose wounds were induced surgically.

MATERIAL AND METHODS

Biological and Technical material

The biological material necessary for this study consisted of plant material, composed of leaves of *Chromolaena odorata* and *Tithonia diversifolia*, and animal material consisting of white albino rats of the wistar strain. The technical equipment consisted of distilled water, cotton, funnel, oven, scales, seals, grinder, gastric tube, graduated syringes, alcohol diluted at 70°.

Extraction and Extraction yield

The crude extract was obtained by extraction with distilled water as solvent (Békro *et al.*, 2007). The extraction yield was calculated after obtaining the extract by the following formula:

$$EY = \frac{\text{Mass of the extract obtained}}{\text{Mass of macerated powder}}$$

Phytochemical screening

The detection tests of the major groups of phytochemicals were carried out on the extracts obtained from water as the extraction solvent. The analytical techniques described in the work of Tona *et al.* (1998) and Longanga *et al.* (2000) were used.

Acute oral toxicity

The experiment was conducted according to guideline 423 of the OECD protocol. Female Wistar strain rats were fasted overnight prior to the experiment. Four (4) groups of three (3) rats distributed at random received increasing doses of the Aqueous extracts (50; 300; 2000; and 5000 mg/kg). The control batch received the solvent for diluting the extracts. Once treated, the animals were observed for 2 hours following the administration of the extract after which they were fed. They were then observed after 4 hours, 8 hours and then 14 days during which the symptoms of intoxication were noted. Dead rats in each lot were counted for LD50 determination.

Healing activity

The wound induction technique used was that of Malone and Morton (1992) with slight modifications. The dorsal fur of each rat was removed 4 cm2 with a razor. The surface was decontaminated with yellow Betadine. Square-shaped wounds with a depth of 0.2 cm and an area of 1 cm2 were induced on the decontaminated surfaces with scalpel blades, sterile forceps, and surgical scissors.

The animals were divided into five groups of 3 rats each, group 1 (negative control) was not treated with any substance after injury induction. Batch 2 (positive control) was treated with Biafine which is the reference substance. Batches 3, 4, and 5 were treated with the

combination of aqueous extracts of *Chromolaena odorata* and *Tithonia diversifolia* at the respective doses of 100, 200 and 400 mg/kg of body weight. The healing activity of the combination was evaluated by a macroscopic technique every 2 days. The evolution of the healing was observed in order to make a visual description and to note the presence or absence of crust, the appearance of the wound, the shrinkage of the wound, the general condition of each rat. The shrinkage of the wound was assessed by measuring the surface when it was not yet completely healed.

RESULTS

Extraction yield

After maceration of 1200 g of vegetable powder from the leaves of *Chromolaena odrata* in 6 liters of distilled water and 780 g of vegetable powder from the leaves of *Tithonia diversifolia* in 4L of distilled water, 74 g of crude extract of *Chromolaena odrata* and 12 g of crude extract of *Tithonia diversifolia*, giving respective extraction yields of 6.2% and 1.5% (Table I).

Table I: Extraction yield of leaves of *C. odorata* and *T. diversifolia.*

Plant species	Mass of vegetable powder (g)	Mass of extract obtained (g)	Extraction yield (%)
C. odorata	12OO	74	6,2
T. diversifolia	780	12	1,5

Phytochemical screening

The highlighting of the large groups of secondary metabolites revealed a positive test for alkaloids, anthocyanins, anthraquinones, flavonoids, polyphenols, saponins, steroids, catechin tannins, triterpenes with the aqueous extract of *Chromolaena odorata* whereas the demonstration of phenols was negative. However, flavonoids were present in large quantities, anthocyanins, anthraquinones, polyphenols, saponins in medium quantity, alkaloids, steroids, catechin tannins, triterpenes, in small quantity (Table II).

Table II: Secondary metabolites of the aqueous extract of *chromolaena odorata.*

Families of metabolites	Aqueous extract of *chromolaena odorata*
Alkaloids	+
Anthocyanins	++
Anthraquinones	++
Flavonoids	+++
Phenols	-
Polyphenols	++

Saponins	++
Steroids	+
catechic tannins	+
Triterpenes	+

+ = presence ; ++ = average presence ; +++ = great presence.

The tests for alkaloids, anthocyanins, anthraquinones, flavonoids, phenols, polyphenols, saponins, steroids, triterpenes were positive with the aqueous extract of *Tithonia diversifolia* while that for catechin tannins was negative. However, Saponins were present in large quantities, Alkaloids, anthocyanins in medium quantity, Anthraquinones, Flavonoids, Phenols, Polyphenols, Steroids, triterpenes in small quantities (Table III).

Table III: Secondary metabolites of the aqueous extract of *tithonia diversifolia.*

Families of metabolites	**Aqueous extract of *Tithonia diversifolia***
Alkaloids	++
Anthocyanins	++
Anthraquinones	+
Flavonoids	+
Phenols	+
Polyphenols	+
Saponins	+++
Steroids	+
catechic tannins	-
Triterpenes	+

+ = presence; ++ = average presence; +++ = great presence.

Acute oral toxicity study

Observation of clinical parameters

During the acute toxicity study, many clinical parameters were observed in order to determine the LD50. The results obtained showed that of all the parameters observed, no anomaly was observed regardless of the batch chosen. The LD50 is therefore greater than 5000 mg/kg (Table IV).

Table IV: Observation of clinical parameters during the acute oral toxicity study.

Observed Paramèters	**Batch 1: control**	**Batch 2: 50 mg/kg**	**Batch 3: 300 mg/kg**	**Batch 4: 2000 mg/kg**	**Batch 5: 5000 mg/kg**
Changing the Coat	Abs	Abs	Abs	Abs	Abs
Eye Modification	Abs	Abs	Abs	Abs	Abs
Modification of the mucous membranes	Abs	Abs	Abs	Abs	Abs
Secretions and	N	N	N	N	N

excretions					
Steps	N	N	N	N	N
Clonic movements	Abs	Abs	Abs	Abs	Abs
Tremors	Abs	Abs	Abs	Abs	Abs
Seizures	Abs	Abs	Abs	Abs	Abs
Diarrhea	Abs	Abs	Abs	Abs	Abs
reaction to sound	N	N	N	N	N
reaction to light	N	N	N	N	N
Mortality	0	0	0	0	0

Abs = Absent ; N = Normal ; 0 = zero.

Effects of the combination on the evolution of the body masses of the batches of rats

The body weights of all rat batches except batch 2 decreased during the acute oral study period. The mass of the control batch decreased from 154.66 g on day zero to 146.33 g on day fourteen, ie a loss in mass of 8.33 g. That of batch 2 increased from 143.33 g on day zero to 148 g on day fourteen, an increase of 4.67 g. That of batch 3 went from 132 g on day zero to 128.66 g on day fourteen, ie a loss in mass of 3.34 g. That of batch 4 increased from 167 g on day zero to 159.33 g on day fourteen, i.e. a loss in mass of 7.67 g and that of batch 5 increased from 160.66 g on day zero to 153.66 g on day fourteen, i.e. a mass loss of 7 g. comparison of body weights of the same batch on day zero and day fourteen revealed significant differences for batches 1, 4, 5 and non-significant for batches 2 and 3 (Figure 1).

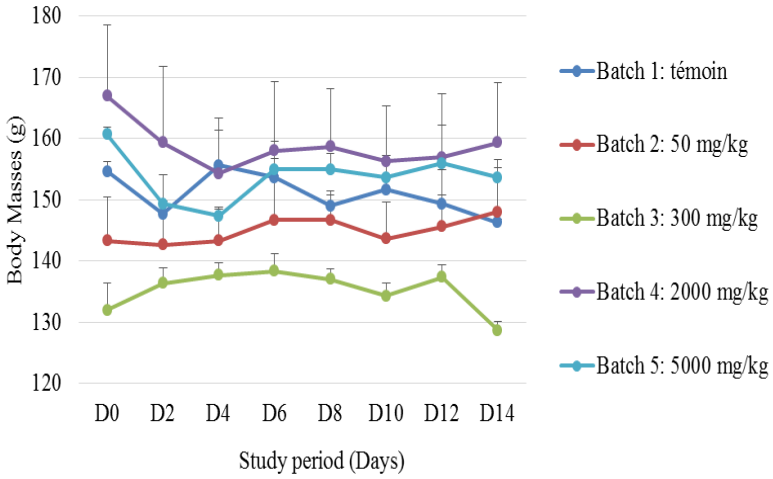

Figure 1: Evolution of the body masses of the batches of rats.

Effects of the combination on the masses of the internal organs of batches of rats

At the end of the acute oral toxicity study period, the masses of the internal organs of the rats were weighed. The highest heart mass (0.58 g) was obtained in batch 1, while the lowest was obtained in batches 4 and 5 (0.53 g). The highest liver mass (4.77 g) was obtained in batch 2,

while the lowest was obtained in batch 3 (3.82 g). The highest kidney mass (0.96 g) was obtained in batch 1, while the lowest was obtained in batch 3 (0.79 g). The highest lung mass (1.99 g) was obtained in batch 5, while the lowest was obtained in batch 3 (1.34 g). Compared to the mass of the heart of the control batch, the masses of the hearts of the rats of batches 2, 3, 4 and 5 were found to be not significantly different (P>0.05). The masses of the livers of the rats of batches 2, 4 and 5 proved to be not significantly different from that of the control batch (P> 0.05) while that of batch 3 was significantly different (p < 0.001). The masses of the kidneys of rats from all batches were not significantly different from that of the control batch (P>0.05) (Figure 2.

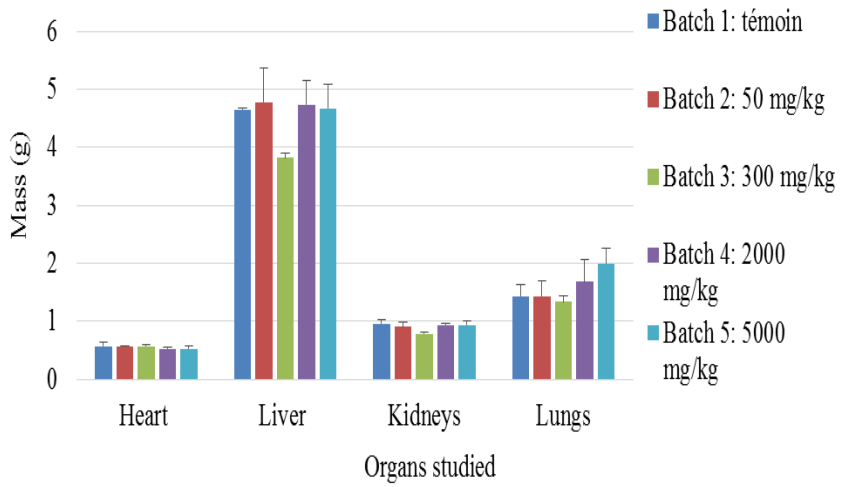

Figure 2: Masses of internal organs of batches of rats.

Influences of the combination of extracts on the blood biochemical parameters of batches of rats

Normal blood creatinine values are in the range of 5 – 11 g/l. the results obtained at the end of this study reveal normal creatinine values in all batches of rats. However, except for batch 2 where the creatinine value is slightly lower than that of batch 1 (control) with very insignificant differences (p < 0.05), the creatinine values of batches 3, 4, 5 were slightly higher than that of batch 1 (control) with non-significant differences (p > 0.05) for batches 3 and 4 and very insignificant (p < 0.05) for batch 5. Normal urea values in the blood are in the range 0.1 – 0.5 g/l. the results obtained at the end of this study reveal values slightly above normal for urea in all batches of rats. Except for batches 2 and 4 where the urea values are slightly lower than those of batch 1 (control) with very insignificant differences (p < 0.05) for batch 2 and non-significant (p > 0.05) for batches 4, the urea values of batches 3 and 5 were

slightly higher than those of batch 1 (control) with very insignificant differences ($p < 0.05$) for batch 3 and non-significant ($p > 0.05$) for lot 5.

Normal ALT values in the blood should not exceed 40 IU/l. this study reveals higher than normal ALT values in all batches of rats. But it should be noted that the ALT value of batch 1 (control) is higher than those of all the other batches with significant differences ($p < 0.001$). Normal AST values in the blood should not exceed 40 IU/l. this study reveals higher than normal values of AST in all batches of rats. Except batch 4 where the AST value is lower than that of batch 1 (control) with significant differences ($p < 0.01$), the AST values of batches 2, 3, 5 were higher than that of the batch 1 (control) with significant differences ($p < 0.001$) (table V).

Table V: Biochemical parameters of batches of rats.

batch number	**Créat (g/L)**	Urea **(g/L)**	**ALAT (UI/L)**	**ASAT (UI/L)**
batch 1 : control	7,43 ± 0,12	0,62 ± 0,04	264,83 ± 62,95	48,33 ± 5,63
batch 2 : 50 mg/kg	6,71 ± 0,33	0,58 ± 0,15	230,41 ± 7,19	62 ± 6,97
batch 3 : 300 mg/kg	7,6 ± 0,95	0,79 ± 0,03	225,16 ± 50,18	52,41 ± 22,35
batch 4 : 2000 mg/kg	7,47± 0,32	0,6 ± 0,04	224,46 ± 39,16	44,99 ± 5,97
batch 5 : 5000 mg/kg	8 ± 0,96	0,63 ± 0,09	246,33 ± 49,07	56,27 ± 12,94

Creat = creatinine; ALT = Alanine amino transferase; AST = Aspartate aminotransferase

Observation of clinical wound parameters during the healing test

Four clinical parameters were observed during this study. The general condition of the wounds appeared normal throughout the study, regardless of the batch chosen. The wounds reddened during the first two days of excision before becoming normal again from the fourth. The presence of crusts on the wounds, as well as the narrowing of the wounds were observed from the fourth day (table VI).

Table VI : Clinical parameters of wounds.

Period	**Parameters**				
	General condition of wounds	**Appearance of wounds**	**Presence of crust on the wounds**	**formation of fleshy buds**	**Shrinking of wounds**
D0	Normal	Blushing	Absent	Absent	No
D1	Normal	Blushing	Absent	Absent	No
D2	Normal	Blushing	present	Absent	Visible
D3	Normal	Blushing	present	Absent	Visible
D4	Normal	Normal	present	visible	Visible
D5	Normal	Normal	present	visible	Visible
D6	Normal	Normal	present	visible	Visible

D7	Normal	Normal	present	visible	Visible
D8	Normal	Normal	present	visible	Visible
D9	Normal	Normal	present	visible	Visible
D10	Normal	Normal	present	visible	Visible
D11	Normal	Normal	present	visible	Visible
D12	Normal	Normal	present	visible	Visible
D13	Normal	Normal	present	visible	Visible
D14	Normal	Normal	present	visible	Visible
D15	Normal	Normal	present	visible	Visible
D16	Normal	Normal	present	visible	Visible
D17	Normal	Normal	present	visible	Visible
D18	Normal	Normal	present	visible	Visible

D = Day

Effects of the combination of extracts on wound healing

The study of wound healing induced by skin excision was conducted over a period of 18 days. The wounds in the negative control group took the longest (18 days) to heal. They were followed by the wounds of batches 2 (Biafine), 3 (extract at 100 mg/kg) and 5 (extract at 400 mg/kg) which lasted 14 days, then by those of batch 4 (extract at 200 mg/kg /kg) which lasted 12 days. However, among the substances administered to wounds lasting 14 days, Biafine was the least effective during the first six days. The plot of its curve is above that of batches 3 and 5, while the most effective substance was the combination of the extracts at a dose of 100 mg/kg. Only the combination of extracts administered at a dose of 200 mg / kg was able to heal the wounds in a period of 12 days, although not being the most effective during the first six days of the study, its effectiveness was greater from the eighth day.

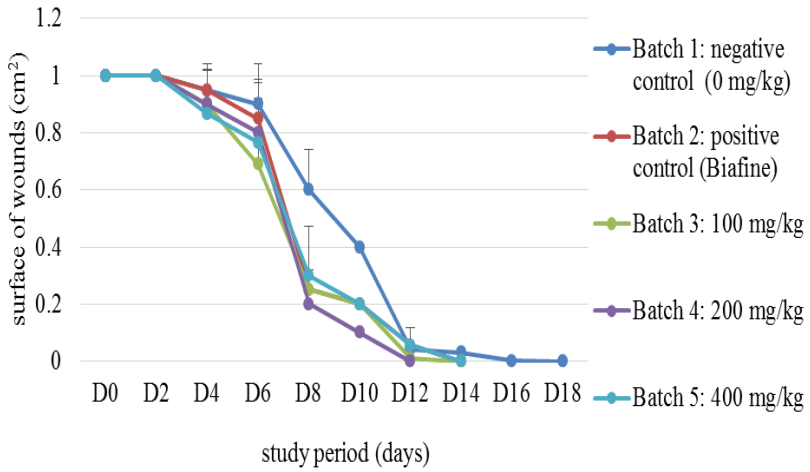

Figure 6 : Evolution of surfaces of the wounds of the batches of rats.

Evolution of the body masses of the batches of rats during the healing test

The body weights of the animals fluctuated widely during the healing study but generally increased regardless of the batch chosen. That of batch 1 (negative control) increased from 137 g on day zero to 172 g on day eighteen, ie an increase in mass of 35 g. That of batch 2 (positive control) increased from 142 g on day zero to 153 g on day eighteen, ie an increase in mass of 11 g. That of batch 3 increased from 129 g on day zero to 148 g on day eighteen, an increase in mass of 19 g. That of batch 4 increased from 142.5 g on day zero to 169 g on day eighteen, an increase in mass of 26.5 g and that of batch 5 increased from 164.66 g on day zero to 169 g on day eighteen, an increase of 4.34 g. the comparison of the body masses of the same batch on day zero and day eighteen using the ANOVA test revealed significant differences regardless of the batch chosen (Figure 7).

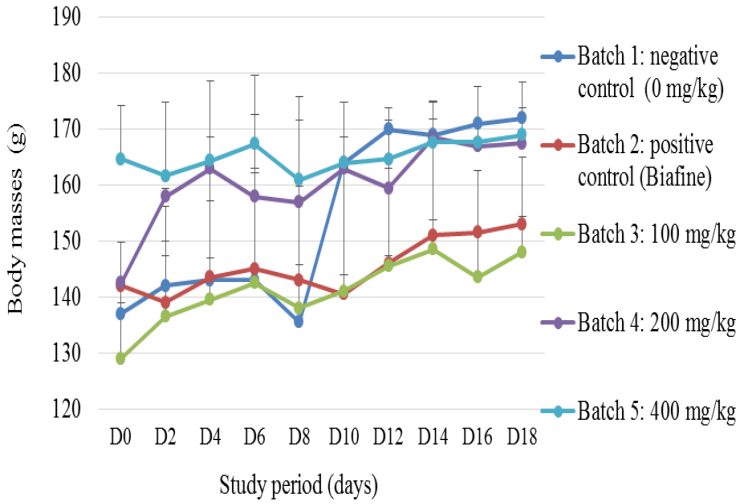

Figure 7: Evolution of the body masses of the batches of rats.

DISCUSSION

In many studies conducted on plants, the yields of extractions have been low. Etame et al. (2017) obtained a yield of 12.14% for their extract. Ekossono (2017) obtained similar yields of 8% and 6.85% for his extracts. These low yields show that *C. odorata* and *T. diversifolia* accumulate few secondary metabolites in the leaves.

The presence of secondary metabolites in plant extracts would be the source of their multiple pharmacological properties. Saponins have a bitter taste. They are heteroside substances with a surfactant property. Alkaloids have anti-ulcer, anti-hypertensive, anti-inflammatory, analgesic properties. Flavonoids have healing, antimicrobial, antimalarial properties and are

also involved in the fight against venereal diseases (Hopkins, 2003). These results are similar to those of Tankeu *et al*. (2020).

The combined aqueous extracts of *C. odorata* and *T. diversifolia* with an LD_{50} lower than 5000 mg/kg are very low in toxicity and good for the formulation of improved traditional medicines, studies of pharmacological activities made and effective doses determined. Sango *et al.* (2008), Ngo Lemba Tom (2011) and Manda *et al.* (2017), found similar results during their work.

The loss of body weights recorded with batches 1, 3, 4 and 5 during the acute oral toxicity study would be due to the fact of the fact of the fact of the fact of the change of the rooms and the composition of the food of the rats rather than the combination of the aqueous extracts of *Chromolaena odorata* and *Tithonia diversifolia* because if this were the case the masses of the control batch which received only distilled water would not fall while that of batch two which received the extracts increased. The result obtained with batch 2 supports that of the observation of clinical parameters by also showing that the combination of aqueous extracts of *Chromolaena odorata* and *Tithonia diversifolia* would be very low in toxicity and good for the formulation of improved traditional medicines. Dibong *et al*. (2018) ; Tankeu *et al.* (2020) found in their acute toxicity work that the weight growth of rats from all batches was increased.

The masses of the livers and kidneys of the batches of rats having been not significantly different from that of the control batch, this would mean that the combination of the aqueous extracts of *Chromolaena odorata* and of *Tithonia diversifolia* would have hepatoprotective and nephroprotective effects. Etame *et al*. (2017), Dibong *et al.* (2018) in their work found similar results.

The normal creatinine values obtained and those of urea very close to normal reflect normal kidney function (Tankeu *et al.*, 2020). These results confirm those of the autopsies after the sacrifice of the rats which revealed that the combination of the aqueous extracts of *Chromolaena odorata* and *Tithonia diversifolia* would have nephroprotective effects.

The higher than normal ALT and AST values obtained in this study reflect abnormal liver function (Tankeu *et al.*, 2020). However, these results confirm those of the autopsies after the sacrifice of the rats which revealed that the combination of the aqueous extracts of

Chromolaena odorata and *Tithonia diversifolia* would have hepatoprotective effects because the levels of ALT and AST were higher in the control batch than in certain batches treated with the combination of extracts and whose effects would rather have contributed to lowering these values. Tankeu et al. (2020), obtained similar results by showing that the combination of aqueous extracts of Picralima nitida and Musanga cecropioides had nephro and hepatoprotective effects in white rats of the wistar strain.

The results of the observation of the clinical parameters of the wounds corroborate those of Ekorong Dang (2018) who worked with the aqueous extract of *Chromolaena odorata* and showed that the reddening reflecting the inflammatory phase of the induction of the wounds was still slightly visible on the third day while the scabs began to appear on the fourth day, thus reflecting the beginning of the shrinkage of the wound surfaces.

The results of this study reveal that all the extracts act by reducing the duration of healing, accelerating haemostasis, decreasing the duration of the inflammatory phase, accelerating the formation of fleshy buds and wound closure. The reduction in the inflammatory phase of the treated batches would explain the early appearance of buds. According to Kishor *et al.* (2011), When the wound is sufficiently cleansed, macrophages and keratinocytes release growth factors that stimulate angiogenesis. This granulation tissue supplies the cells with nutrients to fill the void left by the injury. It constitutes a temporary matrix which allows the migration of endothelial cells, inflammatory cells and fibroblasts to the damaged tissue. The acceleration of the proliferation of granules at the level of the surface of the wounds treated with the extracts at different doses could be explained by the activation of the fibroblasts of the healthy cells in the vicinity of the wound in angiogenesis. This would also explain the re-epithelialization of the wounds treated with the extract before that of the untreated batch. When the buds cover the surface of the wound, the fibroblasts transform into myofibroblasts, which contracting, pull the edge of the edges of the wound towards the center, and close the wounds. According to Stephan *et al.* (2008), Acceleration of the proliferative phase favors the closure of wounds treated with the different doses of the extract compared to that of the wounds of the untreated batch. Blood supply decreases and keratinocytes migrate to granulation tissue.

The increase in body masses recorded in all the batches of rats tested would mean that the induction of wounds in the animals did not prevent their food and water intake. According to Tankeu *et al.* (2020), the increase in the body masses of the animals tested reflects the non-

disruption of their basic metabolism. Rabia (2016), obtained similar results by testing the healing activity of the NJ015 extract in rats.

CONCLUSION

The objective of this work was to study the healing properties of the combination of aqueous extracts of *Chromolaena odorata* and *Tithonia diversifolia*. It appears that the extraction yields are 6.2% and 1.5% respectively for *Chromolaena odorata* and *Tithonia diversifolia*. Tests for the identification of large groups of secondary metabolites proved positive for most of the metabolites tested, regardless of the species chosen. Acute oral toxicity evaluation showed that the combination of aqueous extracts of *Chromolaena odorata* and *Tithonia diversifolia* has an LD50 > 5000 mg/kg, therefore it is classified as non-toxic and good for formulation of improved traditional medicines. The study of wound healing induced by skin excision was conducted over a period of 18 days. Among the substances administered to wounds lasting 14 days, Biafine was the least effective during the first six days. Only the combination of extracts administered at a dose of 200 mg / kg was able to heal the wounds in a period of 12 days, although not being the most effective during the first six days of the study, its effectiveness was greater from the eighth day.

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