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Assessment of *in Vivo* Anti-Ulcer, Gastroprotective Activities, Acute Toxicity and UPLC-ESI-QToF-MS/MS Prediction of *Annickia affinis* (Exell) Versteegh & Sosef (Annonaceae) Stem Bark

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Abstract

Annickia affinis is a medicinal plant traditionally used in Africa against several diseases including peptic ulcers. We conducted this study to evaluate the antiulcer and gastroprotective activities of the ethanolic extract of the stem bark of this plant. The antiulcer activity was evaluated on Wistar rats using 95% ethanol and then administering to them as treatment solutions, the ethanolic extract of the stem bark of the plant and other referenced anti-ulcer drugs over periods of 7 and 14 days. The gastroprotective activity was carried out following the opposite path, the different batches of rats first received one of the treating solutions and an hour later, the ulcer was induced on them and they were finally sacrificed an hour after administring the ulcerogenic solution for the macroscopic and microscopic analysis of their stomachs. Phytochemical screening revealed the presence of alkaloids, saponins, reducing sugars, coumarins, anthocyanins, anthraquinones. UPLC-MS analysis revealed the presence of 16 compounds, notably berberine, berberrubine, canadine, palmatine, jatrorrhizine, columbamine, pseudocolumbamine, 7,8-dihydro-8-hydroxypalmatine, atherosperminine, argentinine, 7-hydroxydehydronuciferine, 7-hydroxydehydronornucierine, Caryophyllene oxide, 1,5-epoxysalvial-4(14)-ene, humulene epoxide II and spathalenol. The administration of the ethanolic extract of the stem bark of *Annickia affinis* at doses of 2000 mg/kg and 5000 mg/kg showed no acute toxicity. The results obtained from the evaluation of pharmacological activities revealed that the ethanolic extract of the stem bark of *Annickia affinis* would have antiulcer and gastroprotective properties comparable to omeprazole and sucralfate at the dose of 150 mg/kg. It appears from this study that the stem bark *Annickia affinis* could be used both in the prevention and in the treatment of gastric ulcers which would justify its use in the traditional pharmacopoeia.

Keywords: *Annickia affinis*, Anti-Ulcerative, Gastroprotective, Ethanolic Extract, Stem Bark.

1.0: INTRODUCTION

Peptic ulcer disease (PUD) is a cosmopolitan disease that affects 10% of the world's population (Praddeepkuman *et al*., 2017). It is defined as a loss of substance from the gastric or duodenal wall reaching deep into the muscularis (College of academics in hepato-gastroenterology, Hepato-gastroenterology digestive surgery, 2015). The causes can be either exogenous factors such as *Helicobacter pylori* infection (The Eurogast Study Group, 1993; Korwin, 2014; College of academics in hepato-gastroenterology, Hepato-gastroenterology digestive surgery, 2015; Praddeepkuman *et al*., 2017), Non steroidal anti-inflammatory drugs (NSAIDs) (French National Society of Gastroenterology, 2000; National Health Insurance Fund, 2006), stress, alcohol, and smoking, or endogenous factors such as hydrochloric acid, pepsin, and reactive oxygen species (ROS) (Rozza *et al*., 2012). Recent studies showed a very low incidence of PUD diagnosed by endoscopy in Europe, particularly in France where it is around 0.2% of the population (Collegiate of university students in hepato-gastro-

enterology, 2014). In Cameroon, the prevalence of PUD diagnosed by endoscopy has significantly decreased from 31.5% to 10.4% between 1990 and 2016, the average age being 48.9 years and the male/female ratio 2.1:1 for duodenal ulcers and 1.2:1 for gastric ulcers (Ankouane *et al*., 2016). On a global scale, the use of modern medicine constitutes a beneficial way of solving health problems through the use of conventional medicines, radiotherapy, and the practice of medical interventions such as surgery, placement of probes and many others.

However, almost 80% of populations depend on traditional medicine for their primary health care (WHO, 2002); this due to the inaccessibility of modern health care facilities, the high costs of conventional medicine but also because of the very high regard given to traditional medicine (Ladoh-Yemeda *et al*., 2016). Going in the same direction, a multitude of plants of Cameroonian biodiversity are used for more than one medicinal virtue. In traditional medicine, several plants are used in the treatment of PUD (Keïta, 2005). *Annickia affinis* is a tree of the *Annonaceae* family. This family is particularly characteristic of paleotropical lowland forests (Versteegh and Sosef, 2007). The tree has been used traditionally in the preparation of several traditional remedies against fever, malaria, urinary tract infections, rheumatism, intercostal pain, liver ailments, tuberculosis, diarrhea, stomach and duodenal ulcers and infections of the stomach. As for the bark powder is applied to wounds, lesions and ulcers of the skin. The bark is said to have aphrodisiac properties (Kémeuzé and Nkongmeneck, 2007; Adesokan *et al*., 2007). The phytochemical study revealed the presence of protoberberine, phenanthrene and aporphine alkaloids, as well as sesquiterpenes (Olivier *et al*., 2015). In addition, several studies have been carried out on *A. affinis* including the *in vivo* study of acute toxicity, the evaluation of the antimalarial, cytotoxic and antioxidant activities of the methanolic extracts of the leaves, stem bark, and roots (Mbosso[a] *et al*., 2023; Ali *et al*., 2023), as well as the subacute toxicity and the assessment of the antibacterial and antifungal activities of the methanolic extract of the stem bark (Mbosso[a] *et al*., 2023). So far, the antiulcer and gastroprotective activities have not been investigated to our knowledge.

In this work, we proposed to make our contribution by studying the *in vivo* acute toxicity, the phytochemical screening, the mass spectrometric dereplication of the secondary metabolites, and the *in vivo* antiulcer and gastroprotective activities of the ethanolic extract of the stem bark of *A. affinis*.

2.0: MATERIALS AND METHODS

2.1: Plant Collection and Identification

The stem bark of *A. affinis* used in this study was collected from Mount Kala (Longitude 30 30' N; Latitude 110 13' E), a village in the Center region, located in the commune of Mbankomo, Department of Méfou-et-Akono.The harvested plant was identified in the National Herbarium of Cameroon by comparison with an authentic sample bearing the identification number 6420/HNC.

2.2: Methodologie

2.2.1: Preparation of Extract

The preparation of the ethanolic extract of the plant was carried out in accordance with the method described by Bidié *et al.,* (2008). Briefly, the bark was collected, cut, dried then ground into fine fibers for extraction. The crushed stem bark (2500 g) was mixed with 4.5 l of 90% ethanol. The mixture was stirred for 48 hours at room temperature (25°C) then filtered three times through cotton and 3 mm Wattman filter paper. The solvent was allowed to evaporate at reduced pressure and at 60°C using a rotary evaporator. Then the crude extract obtained was used for chemical analysis, acute toxicity study and evaluation of antiulcer activity.

2.2.2: Qualitative Phytochemical Analysis of the Extract

The extract was tested for the presence of secondary metabolites groups and classes, namely reducing sugars, alkaloids, saponins, triterpens, steroids, phenols, flavonoids, coumarins, anthocyanidins, tannins and anthraquinones, following common procedures described by other authors (Ronchetti and Russo, 1971; Hegnauer, 1973; Wagner, 1983; Bekro *et al*., 2007).

2.2.3: UPLC-HR-ESI-MS/MS analysis of *A. Affinis* Ethanolic Stem Bark Extract

The ethanolic extract of *A. affinis* stem bark was subjected to UPLC-ESI(+)-MS/MS analysis on a Compact QTOF (Bruker, Bremen, Germany) mass spectrometer coupled to a Dionex Ultimate 3000 UPLC (Thermo Fisher Scientific, Sunnyvale, CA, USA) equipped with a Fortis C18 column (3.0 × 100 mm, 3 µm; Fortis Technologies Ltd, Cheshire, UK) operated at 40°C. A guard column (5 mm × 2.1 mm, 3 µm) with the same stationary phase was placed before the column. Samples were prepared at 1 mg/ml in methanol and 10 µl were injected. The mobile phase consisted of a mixture of water and acetonitrile (MeCN), both adjusted with 0.1% formic acid (FA). These solvents were all of LC-MS grade and obtained from Sigma-Aldrich, Johannesburg, South Africa. The mobile phase gradient was executed with a flow rate of 0.200 ml/min as follows: 0–5 min (H_2O–MeCN, 80:20, *v*/*v*), 5–15 min (H_2O–MeCN–FA, 85:15:0.1, *v*/*v*/*v*), 5–15 min exponential ramp (curve 5) to pure MeCN with a hold of 5 min, followed by 5 min reequilibration.

High-resolution tandem mass spectrometry (HR-MS/MS) data were acquired in the mass range of 50-3000 *m/z*. The parameters of the electrospray ionization source in positive mode were as follows: End plate offset: 500 V, Nebulizer: bar, dry gas: 9.0 L/min, dry temperature: 200°C; capillary voltage: 4.5 kV. Nitrogen was used as nebulizer and dry gas.

Tragmentation spectra were recorded from the 15 most intense peaks per scan (threshold = 4000 cts), with active exclusion for 0.5 min after five spectra and pull exclusion when current intensity exceeded previous intensity by a factor of 2. The collision energy was set to 40 eV with an isolation width of 1.5 *m/z*. Data were recorded as centred and converted to "mzXML" format. The converted raw data were processed in MZmine3 (ver.3.7.0) (Schmid *et al*., 2023) and analysed using a feature-based molecular networking workflow (Nothias *et al*., 2020) on the GNPS online platform (Wang *et al*., 2016). In addition, the processed data were analyzed using SIRIUS5 (ver.5.6.2) for molecular formula determination (Dührkop *et al*., 2019) and the CANOPUS plugin for compound class prediction (Dührkop *et al*., 2021). For MZmine3, mass detection was performed with a noise level of 1E3 for MS1 and 8E1 for MS2 spectra, and chromatograms were constructed using the ADAP chromatogram builder, with a minimum of five consecutive scans, exceeding 5E3 and reaching a maximum of at least 1E4 counts. The *m/z* tolerance was set to 10 ppm. Features were then deconvoluted using the Local Minimum Search module with a chromatographic threshold of 15%, a RT search range minimum of 0.1 min, and a peak duration range of 0-5 min. The Isotopic Peak Finder module was used to assign isotope peaks, taking into account possible elements C, H, N, O, P, S and Cl with an *m/z* tolerance of 10 ppm. Next, the ^{13}C isotope signals were filtered out using the ^{13}C isotope filter module with a RT tolerance of 0.1 min and a *m/z* tolerance of 5 ppm. The Duplicate Peak Filter was used with a RT tolerance of 0.1 min and a *m/z* tolerance of 10 ppm. The feature list was filtered to retain only features between 1.2 and 22 min with a minimum of two features in an ion pattern and feature IDs were reset. Finally, output files (.mgf and .csv) were exported for analysis with GNPS and SIRIUS5. The processed data output from MZmine3 was uploaded to GNPS and a feature-based molecular network workflow was performed using mass tolerances of 0.02 Da. The molecular network was generated with a minimum cosine of 0.7, a minimum of six matching fragments, a maximum of ten edges per node and a maximum of 100 nodes per cluster. Library matching was performed with a minimum of 4 matching fragments and a cosine threshold of 0.7, with all filters disabled. The processed data output from MZmine3 was additionally analysed by SIRIUS5 using default parameters except for an *m/z* tolerance of 15 ppm and consideration of C, H, N, O, P, S, Cl. GNPS and SIRIUS5 results were merged in Python and visualized in Cytoscape (ver.3.9.1) (Shannon *et al*., 2003)

2.2.4: Acute Toxicity Study

The acute toxicity study was performed following the Organization for Economic Co-operation and Development (OECD) protocol directives N°423 (OECD, 1979), with minor changes applied for the chemical products assay and acute oral toxicity, as per other authors. Briefly, the rats distributed in the different batches were treated by single intra gastric administration of a predetermined volume of the extract solution. After gavage, the animals were observed for the first 30 minutes then at 4 hours, thereafter every 24 hours for 14 days to note possible signs of toxicity. The observation focused on various manifestations: tremor, grooming, crowding, reaction to light and sound, appearance of secretion and excretion, mobility, coat, neurovegetative reactions. The animals were weighed every 2 days during the test period.

2.2.5: Preliminary Assessment of the Ulcerogenic Solution

The aim of this test was to ensure the effectiveness of ethanol in inducing gastric ulcers in rats (Lahouaou, 2020). The test was conducted by administering 95% ethanol at 5 ml/kg intragastrically to one batch of 3 rats in order to observe the effects produced on the gastric mucosa of the animals (Lahouaou, 2020). A second batch served as a control and was received distilled water at 5 ml/kg. The rats were deprived of food for 24 h and of water for 2 h before administration of ethanol. One hour after administration of the ulcerogenic solution, the rats were sacrificed. The stomach of each rat was removed, opened along the major curvature using a scissor and washed with physiological solution. Then, each stomach was well spread out on a tablet to better appreciate the ulcerations formed and visible to the naked eye or using a magnifying glass. Only furrows and bleeding points are considered ulcerations, as per Lwoff's technical sheet (Lwoff, 1971). Each stomach was rated according to the following score: No ulcerations = 0; 1 to 2 ulcerations = 1; 3 to 4 ulcerations = 2; More than 4 ulcerations = 3. The ulceration index (UI) was calculated in each batch, with the following formula:

UI = (Sum of ratings × % of stomachs with ulcers)/(Number of animals)

The calculation of the percentage of ulceration was done as described by Nyilimana (2007). A100% ulceration was considered when the sum of the ratings was equal to 12, equivalent to an index of 3. Thus a percentage of ulceration (PU) was drawn using the following formula:

PU = (IU×100)/3

The microscopic examination was carried out by making and reading histological sections.

2.2.6: Assessment of the Antiulcerative Property of the Extract

In order to assess the ulcer-healing capacity of the extract, gastric ulcer was induced in well-fed rats (10 batches; *n* = 4) by administration of 5 ml/kg ethanol solution. After 1 h, the rats were treated with different substances. The ethanolic extract of the stem bark of *A. affinis* was administered, at two weight-doses, namely 150 mg/kg and 300 mg/kg, following the recommendations of Schmeda-Hirschmann and Erdem (2005). Rats treated with the extract were compared to a negative control batch treated with distilled water or

DMSO + Extra virgin olive oil at 5:95 v/v. Positive controls batches were treated with solution of sucralfate 3 g/100 ml (a reference gastric dressing) and omeprazole 20 mg/10 ml (a reference proton pump inhibitors). The solutions were administered once daily to each batch for 7 days (batches 1-5) and 14 days (batches 6-10), regularly between 9 am and 10 am. Each solution was prepared extemporaneously for daily administration. Six hours after administration of the last dose of solution, the rats were euthanized and then gastrectomized. The stomachs were opened along the greater curvature and rinsed with physiological water. Macroscopic and microscopic examinations of the stomachs were carried out as detailed in the preliminary test.

2.2.7: Assessment of the Gastroprotective Activity

In order to assess the ability of the extract to prevent gastric ulcer, the rats were divided into 5 groups (n = 3) and fasted for 24 hours. The solutions from the previous test were administered orally using a feeding tube. One hour after administration of the treatment solutions (ethanolic extract of the stem bark of *A. affinis*, at two weight-doses, 150 mg/kg and 300 mg/kg), ethanol was administered to each rat, at a dose of 5 ml/kg. One hour after administration of the ulcerogenic solution, the rats were euthanized and then gastrectomized. The stomachs were opened along the greater curvature and rinsed with physiological saline for macroscopic and microscopic examinations as previously presented. Calculations of ulceration index and ulceration percentage were performed as detailed in the preliminary test. The percentage inhibition (PI) of gastric ulcer for each treated group was obtained by applying the following formula:

$$PI = \frac{(UIc - UIt) \times 100}{UIc}$$, with:

UIc: Ulceration Index of the control group
UIt: Ulceration Index of the test group

Once the macroscopic analysis of the stomachs was completed, the organs were fixed in 10% formalin solution and preserved in sample jars for the anatomopathological study of the tissues. The histopathological indices were thus determined.

2.2.8: Statistical Analysis

The data from all tests were recorded using MS Excel 2016 and analyzed using GraphPad Prism 8.0.1 software. All results were expressed as means ± SEM values. Statistical analysis was carried out using the one-way ANOVA and two-way ANOVA methods using DUNNETT's parametric test with a significance level of 0.5%.

3.0: RESULTS

3.1: Phytochemical Analysis of the Extract

The dried out ethanolic macerate of *Annickia affinis* stem bark (2500 g) presented as a brown sticky paste of 181.95 g, thereby showing a 7.28% yield. The ethanolic extract of *A. affinis* was tested for the presence of different metabolites. The results (Table 1.0) showed that the extract contained reducing sugars. Some phenolic compounds tested were present (coumarins, anthocyanins and anthraquinones), while other were not (flavonoids, tannins and sterols) were not found. Saponins and polyterpenes, as well as alkaloids were present in the extract.

Table 1.0: Phytochemical analysis of ethanolic extract of *Annickia affinis* stem bark

Phytochemicals	Results
Reducing sugars	+
Flavonoids	-
Tannins	-
Coumarins	+
Anthocyanins	+
Anthraquinones	+
Sterols	-
Saponines	+
Triterpens	+
Alkaloids	+

Caption: (+) = present; (-) = absent.

3.2: UPLC-HR-ESI-MS/MS analysis of *Annickia affinis* ethanolic stem bark extract

The LC/MS analysis of the ethanolic extract of *A. affinis* stem bark revealed the presence of 38 peaks. On these peaks 11 were identified, corresponding to 16 compounds. Among the annotated compounds, twelve alkaloids, and four terpene derivatives were identified (Figure 2.0, Table 2.0).

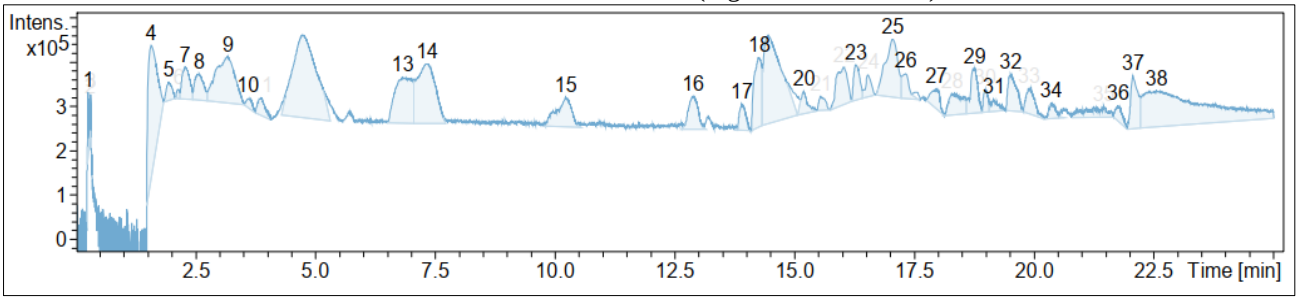

Fig 1.0: Base peak chromatograms of ethanolic extract from *Annickia affinis* stem bark

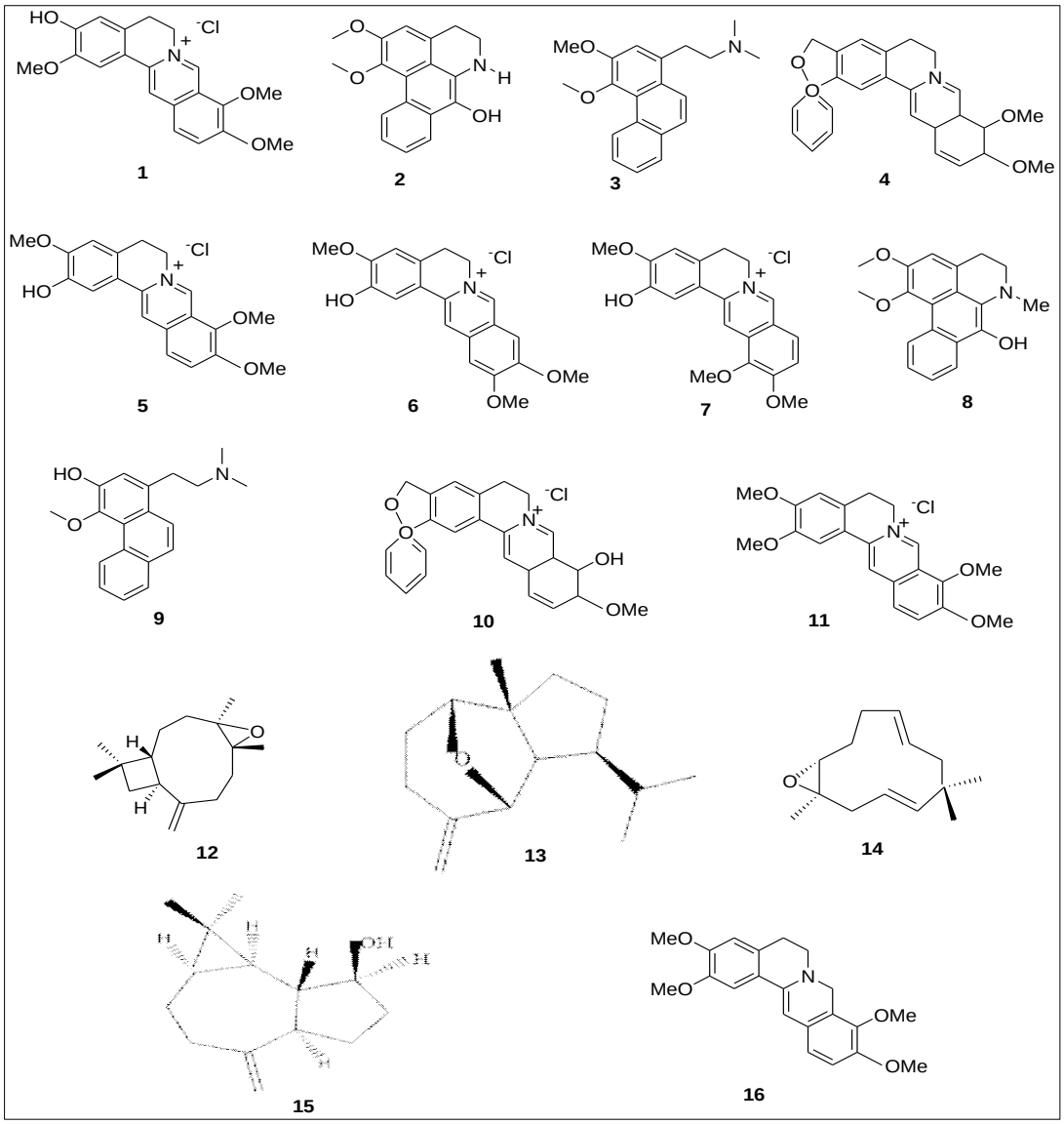

Fig 2.0: Structures of compounds found in the ethanolic extract of *Annickia affinis* stem bark

Table 2.0: LC-MS annotation of the main compounds in ethanolic extract from *Annickia affinis* stem bark.

Peak N°	Structure N°	Pseudo molecular ion	*m/z*	Annotation	Formula	Reference
5	1	$[M+2H]^{\cdot 2+}$	356.1845	Palmatine	$C_{21}H_{25}NO_4$	Olivier *et al*., 2015
6	2	$[M+H_20]^{\cdot +}$	296.1628	7-OH-dehydronornuciferine	$C_{18}H_{18}NO_3$	
7	3	$[M+CH_3\text{-}OH]^{+}$	356.1847	Atherosperminine	$C_{21}H_{26}NO_4$	
8	4	$[M+2H]^{\cdot 2+}$	338.1380	Berberine	$C_{20}H_{20}NO_4$	
9	5	$[M]^{\cdot +}$	338.1380	Jatrorrhizin	$C_{20}H_{20}NO_4$	
*	6			Columbamine		
*	7			Pseudocolumbamine		
11	8	$[M+H]^{\cdot +}$	294.1488	7-OH-dehtempydronornuciferine	$C_{19}H_{20}NO_2$	
4&12	9	$[M+H + CH_3CN]^{+}$	352.1534	Argentinine	$C_{20}H_{22}N_2O_3$	
13	10	$[M+H+H_2O]^{\cdot +}$	340.2585	Berberrubin	$C_{19}H_{18}NO_5$	
14	11	$[M+H]^{\cdot +}$	340.2586	Canadine	$C_{20}H_{13}NO_4$	
17	12	$[M+H_20]^{\cdot +}$	237.1852	Caryophyllene oxide	$C_{15}H_{25}O_2$	
*	13			1,5-Epoxysalvial-4(14)-ene		
*	14			Humulene epoxide II		
*	15			Spathalenol		
34	16	$[M+K]^{\cdot +}$	411.3603	7,8-Dihydro-8-hydroxypalmatine	$C_{21}H_{26}NO_5K$	

**Isomers*

3.3: Acute Toxicity

3.3.1: Physiological Parameters of Rats

The different clinical signs were monitored for 4 h following the intragastric administration of the extract at different doses. The results are compiled in the following table (Table 3.0). The results were similar for all the rats of the different batches. In general, no particular change in behavioral parameters was observed throughout the duration of the test.

Table 3.0: Observation of physical and biological parameters of rats subjected to ethanolic extracts of *Annickia affinis* stem bark

Clinical signs	Control DMSO + Oo	AaSBEt 2000 mg/kg	AaSBEt 5000 mg/kg
Mortality	-	-	-
Fur aspect	N	N	N
Tremors	-	-	-
Mobility	N	N	N
Light and sound sensitivity	N	N	N
Eye modification	-	-	-
Feces aspect	N	N	N
Appearance of neurovegetative reactions	N	N	N
Painful feeling	N	N	N

Caption: *DMSO + Oo: Dimethylsulfoxide + Olive oil; AaSBEt: Ethanolic extract of Annickia affinis stem bark; (-): Absent; N: Normal.*

3.3.2: DL_{50} Determination

Since no deaths were noted after 14 days, the DL_{50} could not be determined. Nonetheless it can be concluded that the $DL_{50} > 5000$ mg/kg for *A. affinis* stem bark ethanolic extract.

3.3.3: Body Mass Variation

The rats were weighed every second day for 14 days and the variations were recorded. The results showed a mild decrease in weight gain in the batch treated with *A. affinis* ethanolic extract, at 5000 mg/kg (Figure 3.0). The weight masses of the different batches increased but not significantly compared to the control batch ($p > 0.3055$).

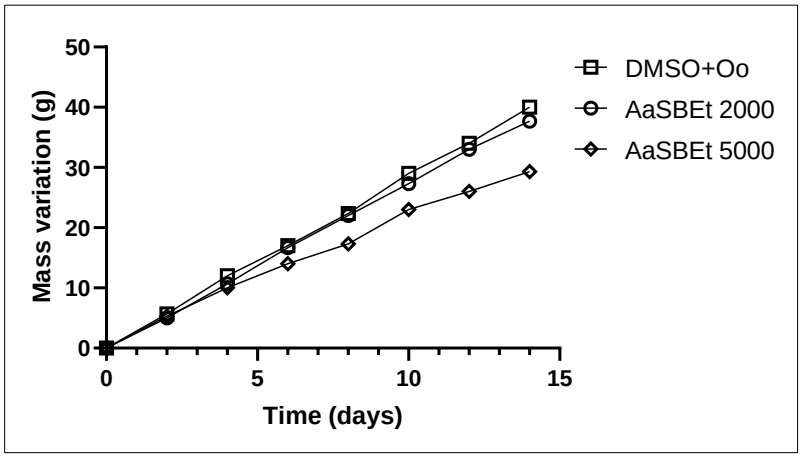

Fig 3.0: 14-Day weight variation of rats following administration of the ethanolic extract of the stem bark of *Annickia affinis*

DMSO + Oo: negative control (dimethyl sulfoxide + olive oil). AaSBEt: Annickia affinis stem bark ethanolic extract, at 2000 and 5000 mg/kg b.w. No statistical difference was obtained in the two tests batches, compared to the negative control ($\alpha = 0.5$).

3.4: Antiulcerative Activity Study

3.4.1: Preliminary Assay

Ethanol (95%) was administered to a batch of rats at 5 ml/kg to ensure its effectiveness in inducing gastric ulcers in rats. The rats were compared to a control batch that received distilled water at 5 ml/kg. The stomach of the rats from the test batch appeared to be highly ulcerated (Figure 3B.0 & 3D.0), with a percentage of ulceration of 88.44%, while the neutral control which showed no ulceration (Figure 4A.0 & 4C.0).

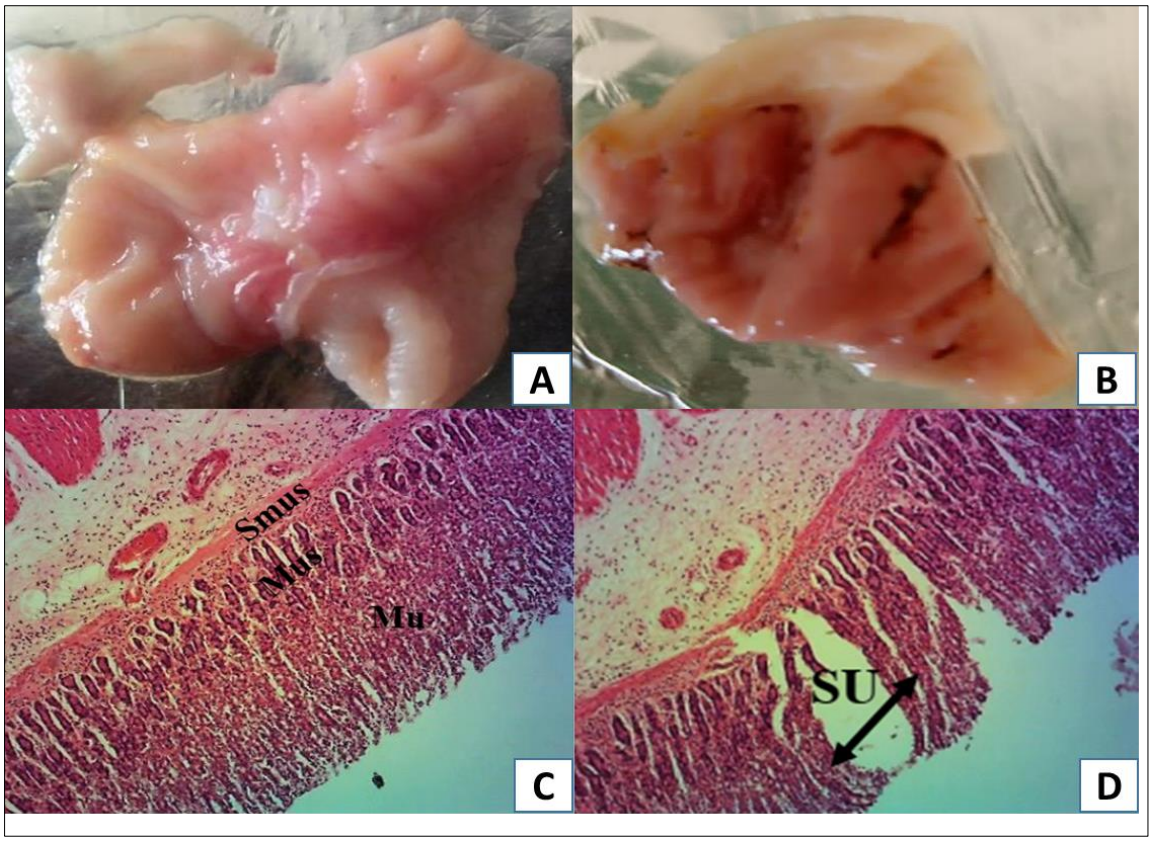

Fig 4.0: Histological section of the stomachs of rats from the preliminary test

(HE, 100X); Mu: Mucosa with gastric glands; Mus: Muscularis mucosa; Smus: Submucosa; SU: Ulcerated surface

3.4.2: 7-Day Outcome on the Extract Activity

Removal and opening of the stomach was performed on the first 5 batches after 7 days of daily administration of the different treatments. The macroscopic aspect of the stomachs of rats from batches having received the extract at 150 mg/kg and 300 mg/kg respectively seemed much less ulcerated compared to the negative control, treated with DMSO + olive oil (Figure 5.0).

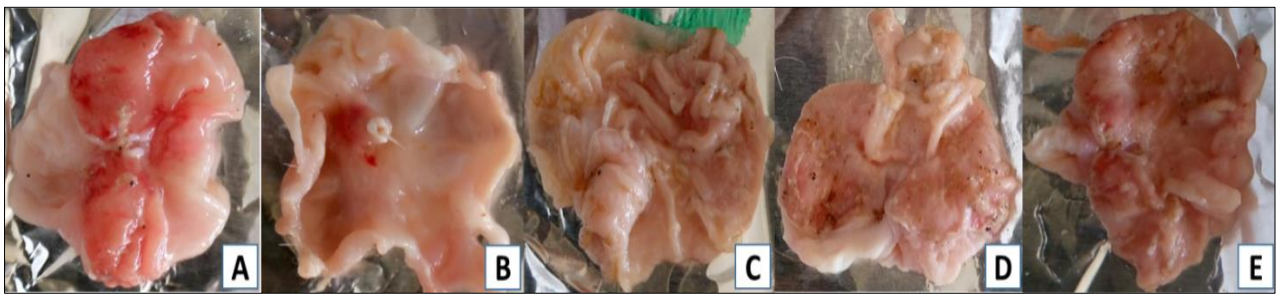

Fig 5.0: Macroscopic aspect of rats stomachs after 7 days of treatment

(A) DMSO + Oo: negative control (dimethyl sulfoxide + olive oil), (B) positive control (sucralfate); (C) positive control (omeprazole), (D) and (E) Test solutions (Ethanolic extract of Annickia affinis at 150 and 300 mg/kg bw, respectively).

Furthermore, the percentage of ulceration (PU) was calculated as previously described. The PUs appeared to be significantly low in both test batches as well as in the positive control batches, compared to the negative control (Figure 6.0). The batch treated with omeprazole showed the lowest percentage of ulceration (18.38%) after 7 days of treatment, followed by the batch treated with the extract at 150 mg/kg bw (33.17%). The PU in the batch treated with a higher dose of the extract (300 mg/kg bw) remained higher than that of the batch treated with the extract at 150 mg/kg bw, but nearly equal to the batch treated with sucralfate (41.33%).

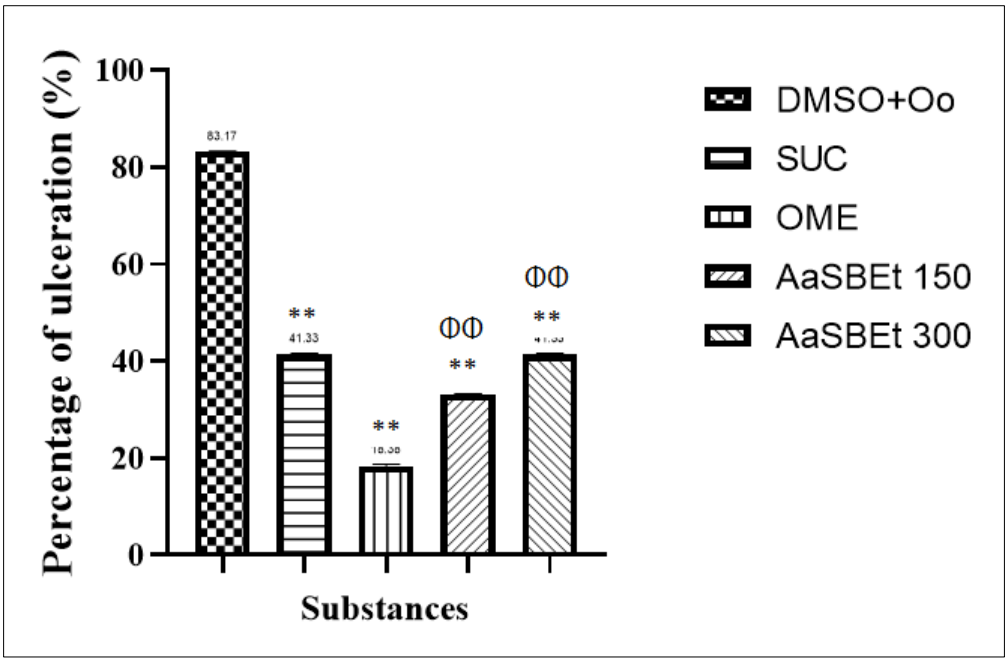

Fig 6.0: Percentage of ulceration after 7 days of treatment

DMSO + Oo: negative control (dimethyl sulfoxide + olive oil); SUC: positive control (sucralfate); OME: positive control (omeprazole); AaSBEt: Test solutions (Annickia affinis stem bark ethanolic extract, at 150 and 300 mg/kg b.w). (**) and (ΦΦ) statistically significant differences, compared to the negative control ($p < 0.01$) and omeprazole ($p < 0.01$), respectively.

3.4.3: 14-Day Outcome of the Extract Activity

Stomachs of rats from the last 5 batches were removed and opened for observation after 14 days of daily administration of the treatments. Figure 7.0 shows the macroscopic aspect of stomach of rats from the different batches and reveals the alteration on the mucosa in comparison to different control groups. The stomachs of rats from batches having received the ethanolic extract of *A. affinis* stem bark at 150 mg/kg and 300 mg/kg appeared much less ulcerated compared to the negative control.

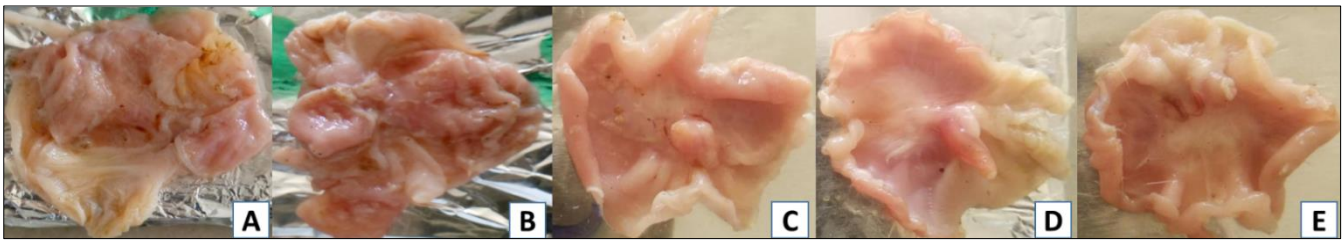

Fig 7.0: Macroscopic aspect of rats stomachs after 14 days of treatment

(A) DMSO + Oo: negative control (dimethyl sulfoxide + olive oil), (B) positive control (sucralfate); (C) positive control (omeprazole), (D) and (E) Test solutions (Ethanolic extract of Annickia affinis at 150 and 300 mg/kg bw, respectively).

The percentage of ulceration (PU) was calculated for each batch. The stomachs of rats from the batches treated with the extract showed a PU significantly lower than that of the control. The batch treated with the extract at 150 mg/kg presented the lowest PU (8.17%) together with the batch treated with omeprazole, followed by the batch treated with the extract at 300 mg/kg (18.38%) together with the batch treated with sucralfate (Figure 8.0). Overall, the PUs were lower compared to those observed after 7 days.

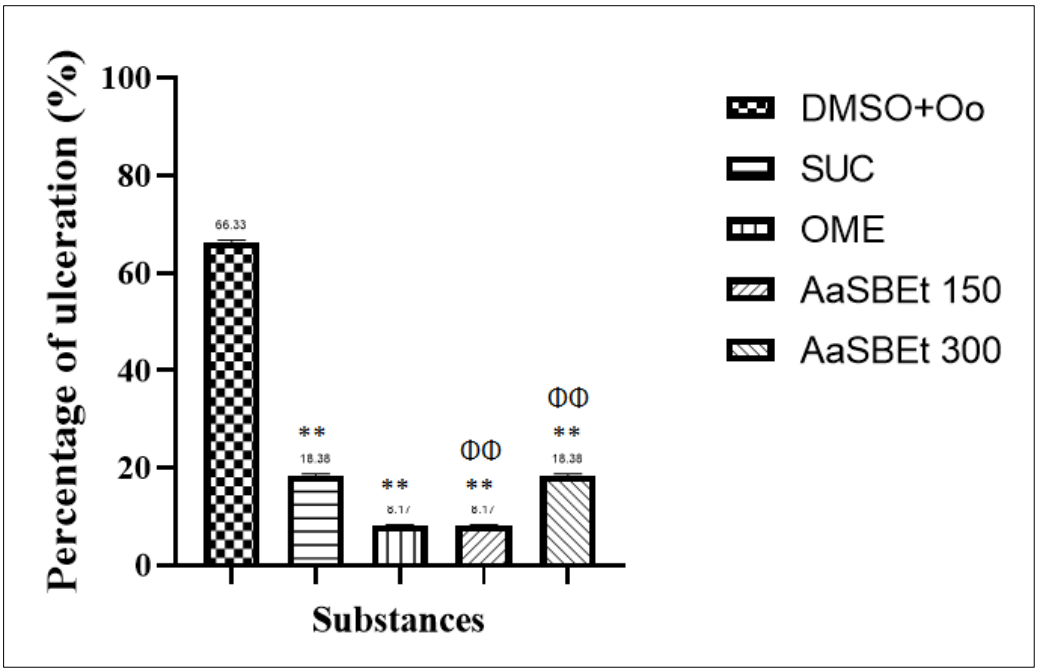

Fig 8.0: Percentage of ulceration after 14 days of treatment

DMSO + Oo: negative control (dimethyl sulfoxide + olive oil); SUC: positive control (sucralfate); OME: positive control (omeprazole); AaSBEt: Test solutions (Annickia affinis stem bark ethanolic extract, at 150 and 300 mg/kg b.w). (**) and (ΦΦ) statistically significant differences, compared to the negative control ($p < 0.01$) and omeprazole ($p < 0.01$), respectively.

3.4.4: Histological Examination of Stomach Mucosas

Microscopic examination of the stomachs confirmed that those having received the ethanolic extract of *A. affinis* stem bark at 150 and 300 mg/kg presented a significantly reduced ulceration surface compared to the negative controls having received DMSO + olive oil à (Figure 9.0). Moreover, the percentage of ulceration appeared lower in the lower dose of the extract (150 mg/kg) than in the higher dose (300 mg/kg).

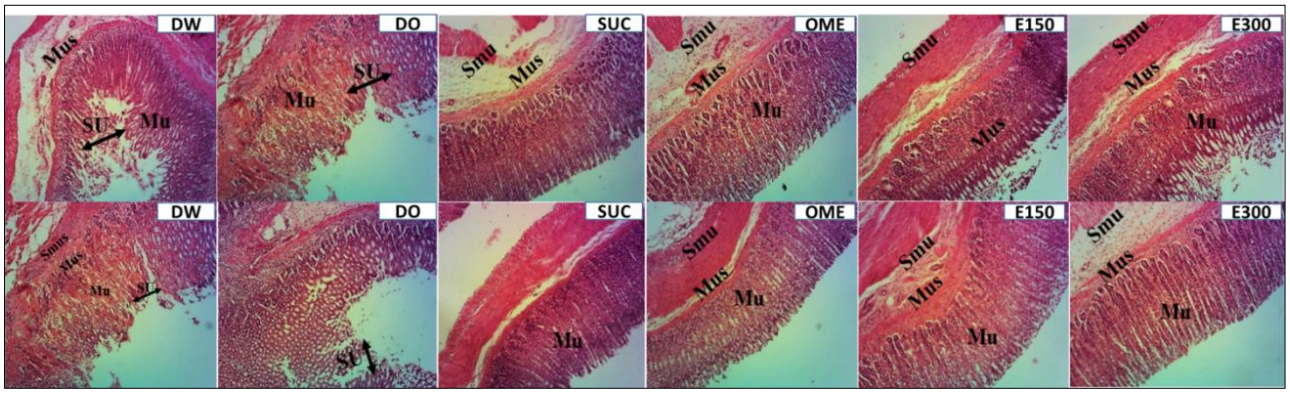

Fig 9.0: Histological assessment of ulcerated stomach mucosas treated with different substances

Mucosas after 7 days (top) and 14 days (bottom) of treatment are shown. Rats were treated with DW: negative control (distilled water); DO: negative control (DMSO + olive oil); SUC: positive control (sucralfate); OME: positive control (omeprazole); E150 & E300: Test solutions (Annickia affinis stem bark ethanolic extract, at 150 and 300 mg/kg b.w); Mu: Mucosa with gastric glands; Mus: Muscularis mucosa; Smus: Submucosa; SU: Ulcerated surface.

3.4.5: Gastroprotective Activity

3.4.5.1: Macroscopic Examination of Rats Stomachs

Figure 10.0 shows the macroscopic appearance of the stomachs of the different batches of Wistar strain rats and highlights the macroscopic alterations of the stomachs of the different batches in comparison with the neutral control batch. The stomachs of rats from batches treated with the ethanolic extract of *A. affinis* stem bark seemed much less ulcerated compared to the controls.

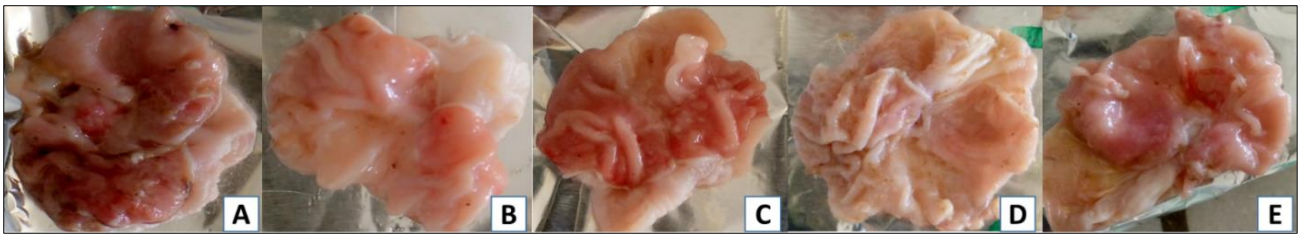

Fig 10.0: Macroscopic aspect of stomachs of rats pre-treated with ethanolic extract of *Annickia affinis* stem bark

(A) DMSO + Oo: negative control (dimethyl sulfoxide + olive oil), (B) positive control (sucralfate); (C) positive control (omeprazole), (D) and (E) Test solutions (Ethanolic extract of Annickia affinis at 150 and 300 mg/kg bw, respectively).

3.4.5.2: Inhibitory Capacity of the Extract against Ulcer Formation

The assessment of rats' stomachs showed a lower percentage of ulceration (14.33%) in the batch treated with the extract at 150 mg/kg, followed by the ones treated with sucralfate and the extract at 300 mg/kg (33.17%). The batch treated with omeprazole showed a high percentage of ulceration (77.33%) while the control batch showed 86.33% of ulceration (Figure 11.0). Thus a higher percentage of inhibition (83.04%) for the extract at 150 mg/kg (Figure 12.0).

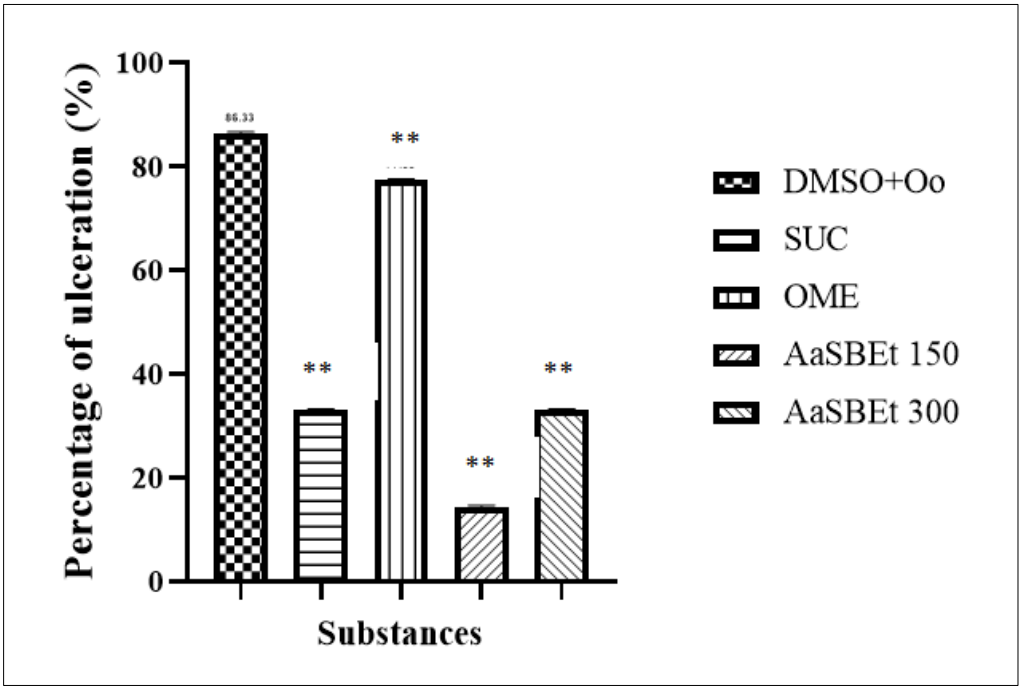

Fig 11.0: Percentage of ulceration in rats pre-treated with ethanolic extract of *Annickia affinis* stem bark

DMSO + Oo: negative control (dimethyl sulfoxide + olive oil); SUC: positive control (sucralfate); OME: positive control (omeprazole); AaSBEt: Test solutions (Annickia affinis stem bark ethanolic extract, at 150 and 300 mg/kg b.w). (**) statistically significant difference compared to the negative control ($p < 0.01$).

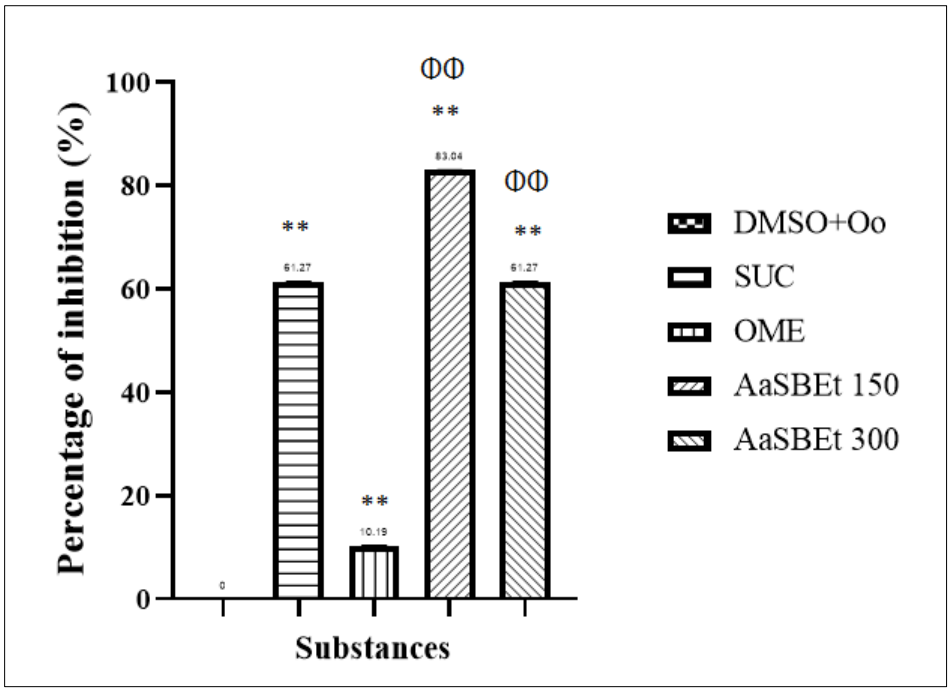

Fig 12.0: Inhibition of ulcer formation in rats by ethanolic extract of *Annickia affinis* stem bark

DMSO + Oo: negative control (dimethyl sulfoxide + olive oil); SUC: positive control (sucralfate); OME: positive control (omeprazole); AaSBEt: Test solutions (Annickia affinis stem bark ethanolic extract, at 150 and 300 mg/kg b.w). (**) and (ΦΦ) statistically significant differences, compared to the negative control ($p < 0.01$) and omeprazole ($p < 0.01$), respectively.

3.4.5.3: Mucosas Histological Examination

Histological examination of the stomachs (Figure 13.0) showed that those having received the plant extracts (E150 and E300) are only slightly ulcerated compared to those having received only distilled water or DMSO + olive oil (respectively DW and DO. Better description from experts.

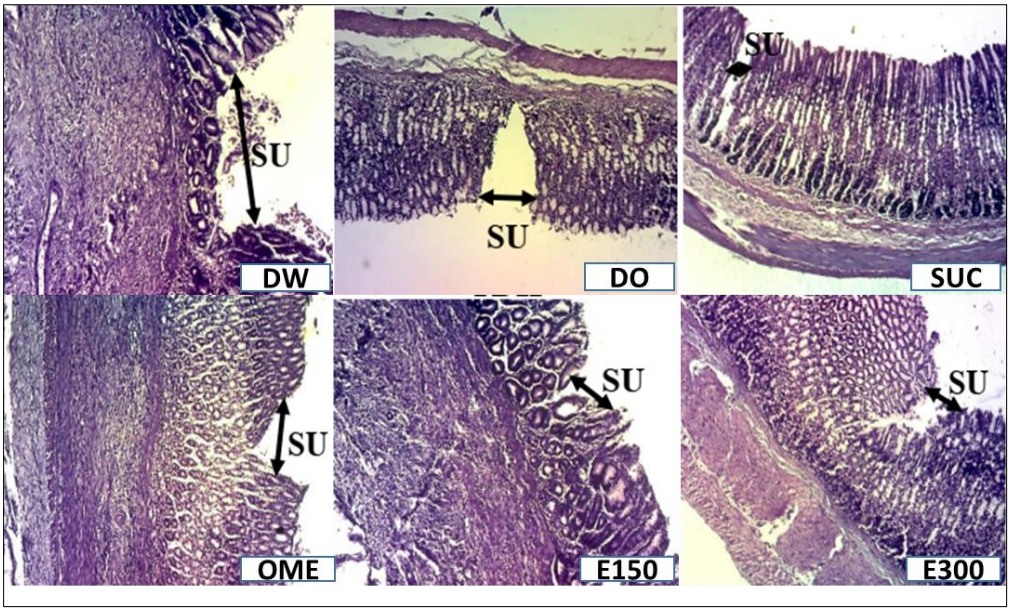

Fig 13.0: Histological assessments of gastroprotective effect of *Annickia affinis* stem bark ethanolic extract.

Rats were treated with DW: negative control (distilled water); DO: negative control (DMSO + olive oil); SUC: positive control (sucralfate); OME: positive control (omeprazole); E150 & E300: Test solutions (Annickia affinis stem bark ethanolic extract, at 150 and 300 mg/kg b.w); the subtances were administered 1h before the ulcerative solution. Mucosas were examined after 1h. SU: Ulcerated surface.

4.0: DISCUSSION

The ethanol extraction yield in this study was 7.28%, has shown to be higher than methanol extraction (Mbosso[a] *et al*., 2023; Mbosso[b] *et al*., 2023; Ali *et al*., 2023) probably due to the polarity of ethanol. Phytochemical screening of the ethanolic extract of *A. affinis* stem bark revealed the presence of alkaloids, saponins, reducing sugars, coumarins, anthocyanins, anthraquinones and polyterpenes and the absence of flavonoids, tannins and sterols. These results are similar to those of Mbosso[b] *et al*., in 2023 which showed the presence of alkaloids, saponins, terpenes, coumarins and anthraquinones and the absence of tannins and flavonoids from the methanolic extract of the stem bark of this plant (Mbosso[b] *et al*., 2023). These results are also close to those of Mbosso[a] *et al*., in 2023 which revealed from methanolic extracts of the bark of this plant the presence of alkaloids, saponins and terpenes and the absence of tannins and flavonoids (Mbosso[a] *et al*., 2023).

The analysis of liquid chromatography coupled with mass spectrometry of the ethanolic extract of the stem bark of *A. affinis* showed the presence of 38 peaks on the chromatogram, 11 of which were identified as the following compounds: Berberine, Berberrubine, Canadine (tetrahydroberberine), Palmatine, Jatrorrhizine, Columbamine, Pseudocolumbamine, 7,8-Dihydro-8-hydroxypalmatine, Atherosperminine, Argentinine, 7-Hydroxydehydronuciferine, 7-Hydroxydehydronornucierine, Caryophyllene oxide, 1,5-Epoxysalvial-4(14)-ene, Humulene epoxide II and Spathalenol; all already isolated by phytochemical study of the bark of this plant (Olivier *et al*., 2015) and some of the stem bark of *A. chlorantha*, another species of the same genus (Olivier *et al*., 2015). This allowed us to justify an adequacy between the chemical composition of our extract and the data of the literature. In addition, these different compounds identified belong to the families of alkaloids and polyterpenes which presence was observed during our phytochemical screening, thus confirming the results of the phytochemical screening with regard to these 2 classes of secondary metabolites. The unidentified compounds could belong to the other classes of compounds.

The administration of the ethanolic extract of the stem bark of *A. affinis* at doses of 2000 mg/kg and 5000 mg/kg showed no significant clinical signs and no case of mortality. This corroborates with the work of Ali *et al*. carried out in 2020 on the methanolic extract of the stem bark of *A. affinis* at the limit doses of 2000 mg/kg and 5000 mg/kg (Ali *et al*., 2015). This result is also similar to that obtained by the work carried out by Tan *et al*., in 2007 who demonstrated that after oral administration of single doses of 1000, 3000 and 5000 mg/kg of aqueous extract from the stem bark of *Enantia chlorantha* to Swiss mice followed by observation for 7 days, no deaths no growth retardation was observed (Tan *et al*., 2007). These results therefore allowed us to consider the evaluation of the antiulcer and gastroprotective activities of the stem bark of this plant.

Ethanol administered to rats orally at a dose of 5 ml/kg caused ulcerations on the stomachs of rats as demonstrated in the preliminary ulceration test. The treatment of ulcerative lesions induced by ethanol with the extract showed a clear reduction in the lesions compared to the control groups. The effectiveness of our extract against these lesions is justified by the presence of 7,8-dihydro-8-hydroxypalmatine, a molecule already isolated from *A. chloranta* and responsible for the antiulcer and gastroprotective properties in this plant according to Tan *et al*., (2000; 2002)

The ulceration percentages of 33.17% and 41.33% obtained after 7 days of treatment at the respective doses of 150 and 300 mg/kg showing that the most effective dose is that at 150 mg/kg with regard to the curative aspect of ulcerative lesions. This could be justified by the fact that this dose of 150 mg/kg is close to that used by Tan *et al*., in 2000 for a maximum inhibition percentage of 53% (Tan *et al*., 2002). The comparison with the percentages of ulcerations obtained for sucralfate and omeprazole suggests that at a dose of 150 mg/kg the extract would have an activity similar to but lower than that of omeprazole but significantly higher than that of sucralfate. The reduction in these percentages after 14 days of treatment would justify the long-term effectiveness of our extract against ulcerative lesions. The percentage of ulceration of the extract at a dose of 150 mg/kg shows maximum activity at this dose with similarity to the activity of omeprazole.

The treatment solutions previously administered to the rats prevented ethanol-induced ulcers significantly ($p < 0.01$) compared to neutral controls. The effectiveness of our extract in this prevention would be justified by the presence of 7,8-dihydro-8-hydroxypalmatine (Tan *et al*., 2000; Tan *et al*., 2002). The inhibition percentages of 83.04% and 61.27% obtained at the respective doses of 150 mg/kg and 300 mg/kg show that the most effective dose is that at 150 mg/kg with regard to the preventative aspect of ulcerative lesions. For this dose, the activity of the extract is significantly higher than those of sucralfate and omeprazole having respective inhibition percentages of 61.27% and 10.19%. This would suggest that the ethanolic extract of the bark of *A. affinis* at the tested doses have a gastroprotective effect greater than that of sucralfate and omeprazole.

This work is the first on the antiulcerative and gastroprotective activities of the ethanolic extract of *A. affinis* stem bark and allowed to confirm its traditional use in the treatment of gastric ulcers. The results obtained constitute an input to the pharmacological data of the species.

5.0: CONCLUSION

At the end of the study, phytochemical screening of the ethanolic extract of *A. affinis* stem bark revealed the presence of alkaloids, saponins, reducing sugars, coumarins, anthocyanins, anthraquinones and polyterpenes and the absence of flavonoids, tannins and sterols. UPLC-MS analysis revealed 12 compounds, notably berberine, berberrubine, canadine (tetrahydroberberine), palmatine, jatrorrhizine, columbamine, pseudocolumbamine, 7,8-dihydro-8-hydroxypalmatine, atherosperminine, argentinine, 7-hydroxydehydronuciferine, 7-hydroxydehydronornucierine, Caryophyllene oxide, 1,5-epoxysalvial-4(14)-ene, humulene epoxide II and spathalenol. The administration of the ethanolic extract of the stem bark of *A. affinis* at doses of 2000 mg/kg and 5000 mg/kg showed no acute toxicity. The ethanolic extract of the stem bark of *A. affinis* showed an antiulcer activity equivalent to that of omeprazole but superior to that of sucralfate and a gastroprotective activity greater than that of sucralfate and omeprazole at the dose of 150 mg/kg. All the observations and results obtained lead to confirm the use the stem bark of *A. affinis* in traditional medicine to care stomach and duodenal ulcers.

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