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EFFECTS OF AQUEOUS STEM BARKS EXTRACT OF *ANTHOCLEISTA SCHWEINFURTHII* (LOGANIACEAE) ON GASTRIC ULCERS AND ON THE ESTROUS CYCLE IN THE RAT

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ABSTRACT

Anthocleista schweinfurthii is traditionally used to treat the gastric ulcers and certain cases of female infertility; these led us to evaluate its gastro-cytoprotective effects and his influence on the estrous cycle. The gastro-cytoprotective potential of the aqueous stem barks extract of *Anthocleista schweinfurthii* was investigated in rats using several ulcer-inducing methods: cold stress water; absolute ethanol and indomethacin. To study the effect of aqueous stem barks extract of *Anthocleista schweinfurthii* (ASEAS) one estrus cycle, 25 female rats with regular estrous cycle were monitored before, during and after extract administration (250mg/kg; 500 mg/kg and 1000 mg/kg). During this period, a vaginal smear was performed to each animal every morning. Estrus cycle duration, ovary and uterus horn weight, ovary cholesterol, endometrial thickness was evaluated. In all cases, oral administration of ASEAS (250 and 500 mg/kg), dose-dependently, prevented gastric lesion formation. Generally, this cytoprotective action was accompanied by increases in gastric mucus production. Indomethacin administration (50mg/kg) reduced mucus production but did not reduce the cytoprotective effect of ASEAS. In rats treated with indomethacin, ASEAS significantly ameliorated the decreased levels of SOD and catalase whereas the increased level of MDA was also improved in stomacal tissue. ASEAS significantly increased the level of ovary cholesterol and the endometrium thickness. Ovarian weight and duration of oestrus cycle showed no significant variation. The gastro-cytoprotetive effects of ASEAS are attributed to enhanced mucus production and antioxidant properties. Furthermore, ASEAS would have estrogenic effects that would strengthen its gastrointestinal cytoprotective properties.

Key words: Gastric ulcer, estrous cycle, Anthocleista , Loganiaceae.

INTRODUCTION

Anthocleista   schweinfurthii is   a   plant belonging to the family of Loganiaceae which account 14 species (Bach *et al.,* 1967). It is a shrub of secondary forests; mean size (8 to 10 m height) sometimes reaching

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30 m high and 70 cm diameter. It has opposed, simple and whole sheets, with reticulate venation (Schmelzer and Gurib-Fakim, 2008). *Anthocleista schweinfurthii* is called "*bépolopolo*" in Douala language (Cameroon) (Etonde and Ekwala, 1987); *Mkungumaji*, *mtambuumwitu* in Lingala (Congo); "*Fafa*", "*bufafaenab*" in Berhaut language (Senegal) (Kerharo, 1974) and "*Abangak*" in Ntumu and Fang languages in Cameroon, Gabon and Equatorial Guinea (Mezui *et al.,* 2015a). *Anthocleista*

185

Mezui Christophe. *et al.* / *International Journal of Phytopharmacology.* 7(4), 2016, 184-195.

schweinfurthii is widely distributed in tropical Africa. This plant is found in secondary forest, forest clearings and also in swampy areas at 400-1800 m altitude (Bach *et al.*, 1967). It is also found in humid soils (Kerharo, 1974). The young leaf juice, powdered root or bark pulp are used to facilitate wound healing (Kerharo, 1974). In Gabon, the *Bapunus* people use this plant to treat of ulcers. In Congo, the decoction of *Anthocleita schweinfurthii* stems barks is used to treat hernia and female sterility. The decoction of the roots is taken for the treatment of stomach ache in women, ovarian diseases, hernia, bronchitis and fever; it is also taken as a purgative and to trigger delivery. In Tanzania, the decoction of roots is taken against malaria, strong abscesses and as vermifuge. The bark of *Anthocleista schweinfurthii* contains traces of alkaloids and the roots contain about 3% .The leaves, bark and roots contain steroids and terpenes. It contains phenolic compounds, tannins, flavonoids, anthocyanins, leucoanthocyanins, quinine derivatives, polysaccharides, glycosides and cardiotonic compounds (Njayou et *al.*, 2008; Ngombe et *al.*, 2010).   The aqueous stem barks extract of *Anthocleista schweinfurthii* (ASEAS) has preventive and healing effects against gastric ulcers (Mezui et al., 2015a). In addition, this plant extract is of low toxicity (Mezui *et al.*, 2015b). Given the fact that, this plant is used to treat stomach ulcers and some cases of female infertility, these led us to evaluate its gastrointestinal cytoprotective effects and its influence on the estrous cycle. The aqueous extract of the barks of the trunk has the preventive effects and curatives against the gastric ulcers (Mezui *et al.,* 2015a). Moreover, this plant extract would be slightly toxic (Mezui *et al.,* 2015b). Taking into account the fact that this plant is used to treat the gastric ulcers and certain cases of female infertility, these led us to evaluate its gastro-cytoprotective effects and his influence on the estrous cycle.

MATERIELS AND METHODS
Preparation of the plant extract
The stem barks of *Anthocleista schweinfurthii* was harvested in July 2014 in South Cameroon region, Vallée du Ntem subdivision. Botanical identification was done in the National herbarium of Cameroon (Yaounde), by comparison with existing voucher specimens N° HNC: 53944. In the laboratory, the barks were cut into pieces, dried under laboratory temperature and ground to powder. 1000g was introduced in 5 liters of distilled water and then boiled on a heating plate for 30 minutes. The resulting solution was filtered using a n° 3 Whatmann filter paper. The filtrate was lyophilized and the resulting brownish solid was used for the pharmacological tests. The resulting material weighed 60 g, giving a percentage yield of 6 % with respect to the powder.

Animals

Male and female Wistar rats (175 ± 25g; 12±1weeks) were used for the experiments. The animals were raised in the animal house of the Higher Teachers' Training College, University of Yaounde 1. They were fed a standard laboratory diet (NAAPCAM SARL, Yaoundé, Cameroon) and given fresh water *ad libitum*. Prior authorization for the use of laboratory animals in this study has been obtained from Cameroon National Ethics Committee (Reg. N. FWA-IRB 00001954). The use, handling and care of animals were done in adherence to the European convention (Strasbourg, 18.III.1986) for the protection of vertebrate animals used for experimental and other purposes (ETS-123), with particular attention to Part III, articles 7,8 and 9.

Anti-ulcer test
Cold stress-induced gastric lesions
Stress-induced gastric ulcers in rats were provoked using the modified method of Tagaki and Okabe (1968). Following 24 hours of food (but not water) deprivation, test rats were given the extract (250 and 500mg/kg) or cimetidine (50 mg/kg) by oral route while control rats received the vehicle. One hour later, the rats were placed in small individual cages and wire immersed in cold water at 21-23∘C up to the level of the xiphoid for 60 minutes. This operation was repeated for six days. They received a normal diet throughout the experiment. On the seventh day of experimentation, the animals were past 3 hours in water. Immediately after withdrawal, they were sacrificed under anesthesia low with ethyl ether. Were the stomachs removed and the gastric lesions Produced Were Measured. Lesion scores were assigned according to the method described by Martin et al. 1993: no ulcer = 0.0; dilation of vessels and small dots of ulcer = 1.0; ulcer less than or equal to 4 mm long = 2.5; ulcer greater than or equal to 5 mm long = 5.0

Absolute ethanol -induced gastric lesions
The rats were deprived of food for 48 h prior to experimentation but all the animals had free access to tap water. The absolute ethanol solution was used to induce ulcers in the gastric mucosa according to the method of (Robert et *al.*, 1979). The animals received the plant extract by oral route, 1 h before they were given the necrotizing solution. They were killed using ether, the abdomen of each opened and the stomachs removed. The ulcers produced in the glandular region of each stomach were measured and scored as earlier described (Tan et al., 1996): no ulcer =0.0; ulcer surface less than or equal to 0.5 mm^2 = 1; ulcer surface greater than 0.5 and less or equal to 2.5 mm^2 = 2; ulcer surface greater than 2.5 and less or equal to 5 mm^2 = 3; ulcer surface greater than 5 and less or equal to 10 mm^2 = 4; ulcer surface greater than 10 and less or equal to 15 mm^2 = 5; ulcer surface greater than 15 and less or equal to 20 mm^2 = 6; ulcer

surface greater than 20 and less or equal to 25 mm^2 = 7; ulcer surface greater than 25 and less or equal to 30 mm^2 = 8; ulcer surface greater than 30 and less or equal to 35 mm^2 = 9; ulcer surface greater than 35 mm^2 = 10.

Indomethacin-induced gastric lesions

Gastric mucosal lesions were induced by the method describe by Pillai *et al* Santhakumari 1984. The test rats were administered the plant extract (250 and 500mg/kg) *per os* while the negative control and normal rats received distilled water (1ml). Those of 5th group (positive control) received by oral route 60 mg/kg of sucralfate (Ulcar®, Laboratoire Adventis 46, quai de la Rapée-75012, Paris, France) (a reference drug). 1h later, all the animals, except normal rats, received the indomethacin solution, 50 mg/kg (Indocid 25 mg, PHr-RBIL Laboratory,-37081, Gottingen, Germany) by oral route. After 4h, under light ether anesthesia, the abdomen of each rat was opened and the stomach removed. The ulcers produced in the glandular region of each stomach were measured. Scores were attributed to the different ulcerated surfaces based on the scale proposed by par Martin et *al*. in 1993. A sample of each stomach was cut and stored frozen for the subsequent antioxidant tests and in formaline 10% for histological cross-sections.

Measurement of mucus production

The mucus covering of each stomach was gently scraped using a glass slide and the mucus weighed carefully using a sensitive digital electronic balance. The same experimenter performed this exercise each time.

Incidence of gastric ulceration (IGU)

Incidence of gastric ulceration (IGU) was determined using the following formula:

$$IGU = \frac{\text{number of ulcerated rats per group}}{\text{total rats of group}}$$

Percentage inhibition (% I)

The preventive effect of the any antiulcer agents used against the severity of ulceration. This was determined with respect to the negative control using the following formula:

$$\% I = \frac{\text{ulcer index of control} - \text{ulcer index of test}}{\text{ulcer index of control}} \times 100$$

Percentage ulcerated surface (% US)

This is equal to the ratio of the total ulcerated surface and the mean value of the pyloro-antral surface (675 mm^2) multiplied by 100 (Tan *et al*., 1997).

$$\% US = \frac{\text{total ulcerated surface (mm2)}}{675} \times 100$$

Measurement of in vivo Antioxidant capacity of Anthocleista schweinfurthii extract

These tests were carried out using homogenate of stomach tissues. The stomach of each rat was weighed; ground (on an ice tray) and homogenised at 20 % in a 25 mM Tris-HCl buffer, pH 7.5. After centrifugation (eppendorf centrifuge) at 6000 tr/min for 30 minutes, the supernatant obtained was conserved at -4°C for the assay of different oxidative stress parameters: lipid peroxidation was assessed by measuring the levels of malondialdehyde (MDA) in gastric tissue samples (Wilbur *et al*., 1949). Quantification of MDA was done using an extinction coefficient of 1.56×106 M/cm and expressed as pmol of MDA per g of wet stomach tissue. Superoxide dismutase (SOD) activity was measured using a standard method proteins, catalase, malondialdehyde and superoxide dismutase (Misra et Fridovish, 1972) and tissue protein was measured using the Biuret method of protein assay (Gornal et al., 1949). Catalase (CAT) was determined (Sinha, 1972) and expressed as µmol of H_2O_2 per min per mg of protein.

Estrous cycle test

To study the effect of aqueous stem barks extract of *Anthocleista schweinfurthii* (ASEAS) on the estrus cycle, 25 female rats with regular estrous cycle were monitored for two weeks. During this period, a vaginal smear was performed on each animal every morning. On the fifteenth day, the 25 female rats were divided into 5 groups of 5 animals each. From the 15th to the 28th day, in addition to the daily realization of vaginal smears, group I rats received distilled water by gavage; Group II and Group III received the aqueous stem bark extract of *Anthocleista schweinfurthii* at doses of 250 and 500 mg/kg, respectively; groups IV and V received orally the ASEAS at the dose of 1000 mg/kg. On the 29th day, after 12 hours of food privation, all rats were sacrificed under low anesthesia with ethyl ether, except the animals of group V (Satellite Group). The ovaries and the uterine horns of each female rat were collected and then weighed. An ovary from each rat was kept frozen at - 4°C for the determination of cholesterol concentrations. The uterine horns were kept in 10% formaldehyde for histological analysis. From the 29th to 42nd day, group V did not receive the extract but vaginal smears were performed each morning. At 43th day, the rats of the satellite group were sacrificed under low anesthesia with ethyl ether.

RESULTS
Anti-ulcer activity
Cold stress-induced gastric lesions

Table 1 shows that the ASEAS reduced gastric ulcers induced by stress. A significant reduction (p <0.05) of the ulcer index was caused by the extract at doses of 250 mg/kg and 500 mg/kg. The ulcer index

187

Mezui Christophe. *et al.* / *International Journal of Phytopharmacology.* 7(4), 2016, 184-195.

increased from 2.51 for the control group to 1.43 ± 0.19 and 1.09 ± 0.30 for the 250 mg/kg and 500 mg/kg doses of extract, respectively. This reduction in ulcer induction corresponds to 43.02% inhibition and 56.57% for the 250 mg/kg and 500 mg/kg doses of extract, respectively. The inhibition of ulcer induction was accompanied by a significant increase (p <0.01) in the mucus secretion, from 77.93 ± 8.38 (control group) to 96.44 ± 4.38 mg and 119.71 ± 4.37 mg for the doses extract of 250 mg/kg and 500 mg/kg, respectively. Cimetidine (50 mg/kg), the reference product, gave greater inhibition than the extract.

Absolute ethanol-induced gastric lesions

ASEAS and sucralfate did not significantly reduce the ulcer index induced by absolute ethanol. However, the ulcerated surface presented a significant decrease from 7.77% (control group) to 3.36% and 2.44% for the 250 mg/kg and 500 mg/kg doses of extract, respectively. This reduction of the ulcerated area was accompanied by significant (p <0.05) increase in mucus production which at 500 mg/kg dose of extract (Table 2).

Indomethacin-induced gastric lesions
Effect of ASEAS on lesions in gastric mucosa of rats subjected to indomethacin-induced gastric ulcers

Indomethacin (50 mg/kg *per os*) caused gastric lesions in rats with an incidence (IGU) of 1. The extract of *Anthocleista schweinfurthii* caused a significant decrease in the GUI from 1 to 0.80 and 0.20 for the 250 mg/kg and 500 mg/kg doses of extract, respectively. This decreased incidence was accompanied by a significant reduction of the ulcer index (UI) which decreased from 3.96 ± 0.44 in the control group to 1.40 ± 0.48 and 0.50 ± 0.50 for extract doses of 250 and 500 mg/kg, respectively, corresponding to 64.64% and 87.37% inhibition, respectively. Sucralfate (60 mg/kg) resulted in a complete inhibition of ulcers. This inhibition of gastric ulcers was not accompanied by a significant increase in mucus production (Table 3).

Effects of the ASEAS on the microscopic aspect of the stomach mucosa

Microscopic analysis showed that indomethacin (50 mg/kg) induced significant gastric mucosal destruction. Meanwhile, *A. schweinfurthii* extract (250 mg/kg and 500 mg/kg) and sucralfate (60 mg/kg) protected the gastric mucosa against indomethacin-induced destructions. The rats pre-treated with these drugs (sucralfate and extract) presented a normal mucosal tissue, similar to the mucosa of normal rats (Figure 2).

Effects of the ASEAS on some antioxidant parameters

Rats that received indomethacin (50mg/kg) showed a high level of malondialdehyde (3.13 ± 0.31 nmol/g of protein) in the stomach tissue compared to normal rats (2.58 ± 0.11 nmol/g of protein). The extract of *A. schweinfurthii* (500 mg/kg) provoked a significant (P <0.05) decrease in tissue malondialdehyde levels (1.35 ± 2.02 mmol/mg of tissue) compared with the negative control. The decrease of malondialdehyde was accompanied by a significant increase of superoxide dismutase and catalase levels in rats treated with the extract of *Anthocleista schweinfurthii*. The negative control group showed a significant (p <0.01) decrease of superoxide dismutase compared to the normal group. The superoxide dismutase concentration increased from 40.34 ± 4.32 U/g of protein in the negative control group to 200.27 ± 6.15 and 280.62 ± 4.26 U/g of protein in the rats treated with the extract at doses of 250 and 500 mg/kg, respectively. Sucralfate (60 mg / kg) did not induce a significant increase in SOD compared to normal rats. In rats treated with the extract (250 and 500 mg/kg), the concentration of catalase increased significantly (p <0.05) compared to normal rats and negative control groups (Table 4).

Estrous cycle test
Effects of aqueous extract of the bark of *Anthocleista schweinfurthii* on vaginal smears in rats

The vaginal smears carried out daily in female rats revealed an alternation of the types of cells present in the vagina both in extract-treated and control female rats. Alternatively, the following cells were observed:

Large cells having a rounded shape and having a core or not; it is the keratinized epithelial cells. The prevalence of these keratinized cells characterizes the proestrus phase (Figure 2 A).

Anucleated regularly shaped cells, are the cornified cells. Majority their presence indicates the phase of oestrus (Figure 2B). Sometimes these cornified cells were associated with polymorphonuclear cells. This combination of cornified cells and polymorphonuclear characterizes the metestrus phase (Figure 2 C).

In addition, a predominance of polymorphonuclear leukocytes was also observed in the vagina, thus characterizing the diestrus phase (Figure 2D).

These different phases of the estrous cycle (proestrus, estrus, diestrus and metoestrus) were observed with the same rotation as well in female rats treated with the extract as in the control group. No changes were observed after two weeks following discontinuation of the treatment.

Effects of aqueous extract of the bark of *Anthocleista schweinfurthii* (As) on the term of the estrous cycle in female rats

The observation of the four phases of the oestrous cycle made it possible to determine the duration of an oestrous cycle. Table 5 shows that the duration of the oestrous cycle did not present any significant difference between the control group and groups treated

with the extract. For each group, the cycle duration did not present any significant variation before and during the treatment. Moreover, the cycle duration remained constant during the two weeks following discontinuation of the treatment.

Effects of the ASEAS on ovarian weight, uterine weight, uterine endometrial thickness and the content of cholesterol from ovary

Table 6 shows that the extract of *A. schweinfurthii* led to a significant increase (p <0.05) in the

relative weights of the uterus. The thickness of the uterine endometrium increased significantly (p <0.01) and dose-dependently in rats treated with the extract (Table 6 and Figure 3). The cholesterol content of the ovaries also showed an increase that was significant (p <0.05) for the 500 mg/kg and 1000 mg/kg doses of extract. The satellite batch shows that the significant increase in weight of the uterus, endometrial thickness and ovarian cholesterol caused by the extract of *A. schweinfurthii* remained irreversible two weeks after discontinuation of treatment.

Table 1. Effects of stem bark aqueous extract of *A. schweinfurthii* on gastric lesions induced by cold stress water in rats

Treatment	Dose (mg/kg)	N	IGU	UI (mean ± SEM)	%US	I (%)	Mucus (mg) (mean ± SEM)
Control	-	5	1	2.51 ± 0.08	1.72	-	77.93±8.38
A. s. extract	250	5	0.80	1.43 ± 0.19*	0.52	43.02	96.44±4.38
A. s. extract	500	5	0.60	1.09 ± 0.30**	0.17	56.57	119.71±4.37**
Cimetidine	50	5	0.80	0.80 ± 0.20***	0	68.12	91.30±5.24

IGU: incidence of gastric ulceration; MUS: mean ulcer score; %US: percentage of ulcerated surface; UI: ulcer index; N: number of rats; UI: ulcer index; %I: Percentage inhibition;As= *Anthocleista schweinfurthii*; *p<0.05 statistically significant relative to negative control **P<0.01 statistically significant relative to negative control, ***P<0.001 statistically significant relative to negative control; N: number of rats.

Table 2. Effects of stem bark aqueous extract of *A. schweinfurthii* on gastric lesions induced by absolute ethanol

Treatment	Dose (mg/kg)	N	IGU	UI (mean ± SEM)	%US	I (%)	Mucus (mg) (mean ± SEM)
Control	-	5	1	4.86 ± 0.45	7.77	-	52.81±6.92
A. s. extract	250	5	0.80	3.50 ± 1.05	3.36	27.98	68.60±6.47
A. s. extract	500	5	0.80	2.84 ± 0.76	2.44	41.56	87.07±3.58**
Sucralfate	60	5	1	3.06 ± 0.29	2.34	37.03	46.19±3.77

IGU: incidence of gastric ulceration; MUS: mean ulcer score; %US: percentage of ulcerated surface; UI: ulcer index; N: number of rats; UI: ulcer index; %I: Percentage inhibition; As= *Anthocleista schweinfurthii*; **P<0.01 statistically significant relative to negative control; N: number of rats.

Table 3. Effects of ASEAS on gastric lesions induced by indomethacin in rats

Treatment	Dose (mg/kg)	N	IGU	UI (mean ± SEM)	%US	I (%)	Mucus (mg) (mean ± SEM)
Normal rat	-	5	0	0	-	-	100.66±10.88
Control	-	5	1	3.96 ± 0.44	3.77	-	65.01±7.27
A. s. extract	250	5	0.80	1.40 ± 0.48**	1.4	64.64	73.28±7.55
A. s. extract	500	5	0.20	0.50 ± 0.50***	0.5	87.37	73.60±5.51
Sucralfate	60	5	0	0***	0	100	67.93±7.10

IGU: incidence of gastric ulceration; MUS: mean ulcer score; %US: percentage of ulcerated surface; UI: ulcer index; N: number of rats; UI: ulcer index; %I: Percentage inhibition; As= *Anthocleista schweinfurthii*; **P<0.01 statistically significant relative to negative control,***P<0.001 statistically significant relative to negative control.

Table 4. *In vivo* antioxidant capacity of the aqueous stem bark extract of *Anthocleista schweinfurthii* in rats subjected to indomethacin-induced ulceration

Treatment	Dose (mg/kg)	Malondialdehyde (*nmol/g of protein*)	Superoxide dismutase (*U/g of protein*)	Catalase(*μmolof* H_2O_2/min/ g of protein)
Normal rat	-	2.58 ± 0.11	120.21 ± 5.12	64.12 ± 7.30
Negative control	-	3.13 ± 0.31	40.34 ± 4.32§§	44.52 ± 6.21

A.s extract	250	2.68 ± 0.48	200.27 ± 6.15*§	110.08 ± 25.23**§
A.s extract	500	1.35 ± 0.20*	280.62 ± 4.26**§§	148.47 ± 5.65***§§
Sucralfate	60	3.08 ± 0.30	160.14 ± 4.17*	54.31 ± 6.17

Values represent Mean ± S.E.M. *P<0.05, statistically significant relative to negative control ***P<0.001 statistically very significant relative to negative control, *P<0.05, statistically significant relative to negative control, §P<0.05 statistically significant relative to normal rat, §§P<0.01 statistically very significant relative to normal rat, Number of rats N= 5.

**Table 5. Effects of the extract of *Anthocleista schweinfurthii* (As) over the duration of estrus cycle**

Treatment Schedule	Control	A.s 250 mg/kg	A.s 500 mg/kg	A.s 1000 mg/kg	satellite
Before treatment	5.06±0.05	4.99±0.06	4.80±0.04	5.06±0.05	4.86±0.04
During treatment	4.83±0.05	4.91±0.05	5.10±0.04	5.13±0.03	5.04±0.04
After treatment	-	-	-	-	5.00±0.05

Table 6. Effects of the extract of *Anthocleista schweinfurthii* on ovarian weight, uterine weight, uterine endometrial thickness and ovarian cholesterol content

| Treatment	Dose (mg/kg)	N	Ovary weight (%)	Uterus weight (%)	Endometrial thickness (µm)	Cholesterol (mg/dL)
Control	-	5	0.03±0.00	0.14±0.00	2.16±0.18	155.55±0.36
A. schweinfurthii	250	5	0.03±0.00	0.20±0.01*	4.30±0.32**	159.30±0.86
A. schweinfurthii	500	5	0.03±0.00	0.19±0.01	5.04±0.25**	178.90±0.48*
A. schweinfuthii	1000	5	0.03±0.00	0.25±0.01**	5.74±0.31***	193.20±1.72**
Satellite (*A. schweinfurthii*)	1000	5	0.03±0.00	0.25±0.01**	5.96±0.10***	192.30±1.34**

Values represent Mean ± S.E.M. *P<0.05, statistically significant relative to negative control, **P<0.01 statistically significant relative to negative control, ***P<0.001, statistically significant relative to negative control.

**Fig 1. Histological presentation of the acute indomethacin-induced gastric ulcer.**
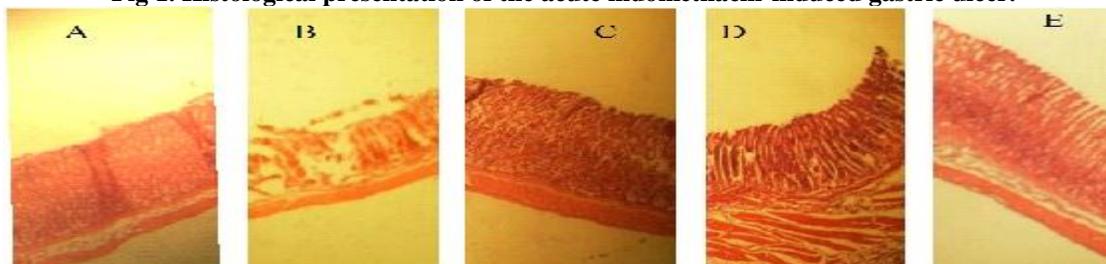
(**A**) Histological section of normal rat stomach showing gastric mucosa, (**B**) Histological gastric sections of negative control rats, showing the superficial loss of substance and glandular destruction; (**C**) Rats treated with sucralfate (60 mg/kg), (**D**) rats treated with 250mg/kg extract and (**E**) rats treated with 500mg/kg extract showed healthy mucosa without glandular destruction.

Fig 2. Photograph of the estrous cycle phases (400 ×, Giemsa staining)
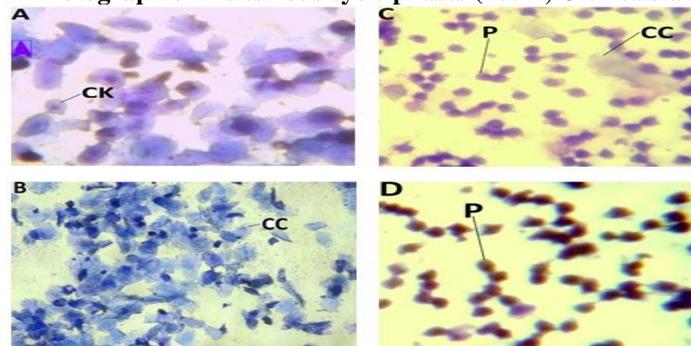
A = Proestrus, B = estrus, C= metaestrus ; D = diestrus. (CK = keratinized cells, CC= cornified cells; P = polymorphonuclear).

Fig 3. Effects of *Anthocleista schweinfurthii* extract on endometrial thickness (400× haematoxylin - eosin). St: stroma; En: Endometrium; Lu: light.
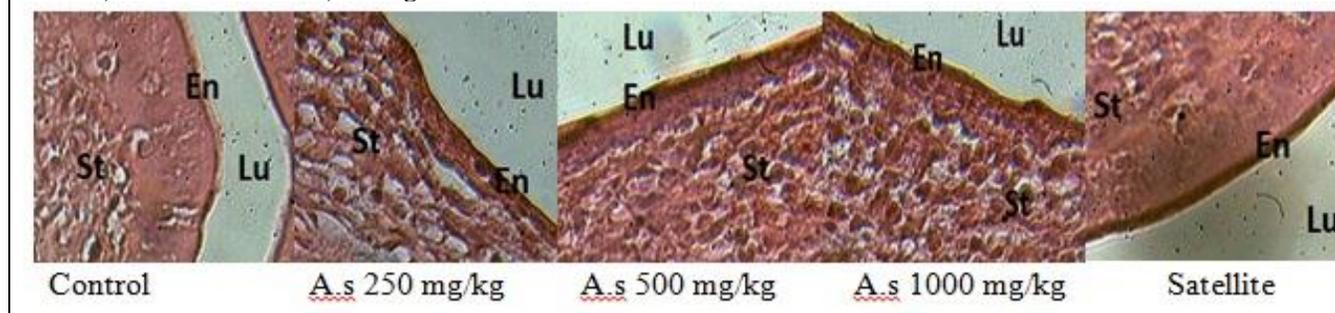
DISCUSSION

The severity of ulcers observed in the control group was due to the fact that cold stress caused increased secretion of gastric acid by vagus nerve stimulation. This nerve induces the secretion of gastric acid through its chemical mediator (acetylcholine) which binds to muscarinic receptors on the membranes of the parietal cells and histamine-secreting cells. The increase in the hydrochloric acid secretion is due to the synergistic action of acetylcholine on the histamine-secreting cells and the parietal cells (Panda and Sonkamble, 2012). Besides the increase in gastric secretion, stress causes decreased mucus production and induces vasoconstriction in the microcirculation of the gastric mucosa (Paul *et al.,* 2003). In rats treated with *A. schweinfurthii* extract (250 and 500 mg / kg), a significant reduction ($p < 0.01$) of the ulcerated surface relative to the control group was obtained. This reduction of ulcerated surface was accompanied by a significant decrease ($p < 0.01$) of the ulcer index (Table 1). These results show that the aqueous extract *Anthocleista schweinfurthii* has a cytoprotective effect on the gastric mucosa. This would be justified by the fact that the aqueous extract of *A. schweinfurthii* inhibits the Na^+/K^+ ATPase pump (Ngo mbe et al., 2010). However, the output of the H^+ ions (by H^+/K^+ ATPase pump) to the gastric lumen is coupled to the input of the K^+ ions. These K^+ ions return to the gastric lumen through the Na^+/K^+ pump (Marieb, 1999). If the pump is inhibited, the secretion of HCl will not take place. Cimetidine (50 mg/kg) significantly reduced ($p < 0.01$) the ulcer index compared with the control group, with 68.12 % inhibition. This may be explained by the fact that cimetidine (reference molecule) inhibits the secretion of hydrochloric acid. It blocks the H_2 histamine receptors, allowing a significant reduction in gastric acidity (Willoquet *et al.,* 2011). However, a previous study on *Anthocleista schweinfurthii* aqueous stem barks extract using the pylorus ligation model showed that this extract does not possess gastric anti-secretory activity (Mezui *et al.,* 2015b). This extract would therefore ensure the gastric cytoprotection by mechanisms other than the reduction in gastric acidity. The aqueous extract of the bark of *A. schweinfurthii* increased significantly ($p < 0.01$) the mucus weight relative to the control group. This significant increase mucus weight would be one of the mechanisms by which the *Anthocleista schweinfurthii* extract can protect the gastric mucosa. The mucus forms a barrier layer which protects the gastric mucosa against the H^+ ions reflux into the gastric wall, and can protect the gastric mucosa against ulcerogenic solutions (Bernier *et al.*, 1986). Previous work suggested that the extract of the bark of *A. schweinfurthii* stimulates mucus secretion *via* endogenous prostaglandin E_2 and by direct action on mucus cells (Mezui *et al.,* 2015b). The aqueous extract of the leaves of *Ocimum suave* also led to a gastric cytoprotection accompanied by significant production ($p < 0.05$) of mucus in rats subjected to cold water stress (Tan *et al.,* 2013). In addition to the increased secretion of gastric acid, stress stimulates the production of active oxygen species (AOS) (Tan *et al.,* 2013). These AOS cause lipid peroxidation of cell membranes resulting in lesions (Panda and Sonkamble, 2012). The extract of *A. schweinfurthii* therefore would have antioxidant properties.

The pathogenesis of gastric ulcers induced by absolute ethanol is very complex. The absolute ethanol directly attacks the cells of the gastric mucosa causing necrosis. It reduces the secretion of bicarbonates and mucus, alters the composition of mucus glycoprotein, and causes the rupture of the glandular cells thereby releasing the acid hydrolases. Absolute ethanol stimulates the release of cell mediators such as histamine and leukotriene C_4 which act on the micro gastric vasculature causing vasoconstriction. These effects of ethanol cause a series of events leading to the destruction of the cells of the mucosa and submucosa (Oates and Hakkinen, 1988). Moreover, absolute ethanol causes a retro-diffusion of the acids through the gastric mucosa (Gharzouli *et al.,* 1999). The ethanol metabolism releases superoxide anion and other free radicals (Elango *et al.,* 2012). The high aggressiveness of absolute ethanol and the complex mechanisms that trigger it to induce ulcers explain the reduction in gastro-cytoprotective effect of the extract of *A. schweinfurthii* in the rats which received absolute ethanol. The ulcer index was 4.86 ± 0.45 in the control group, 2.84 ± 0.76 in extract-treated rats (500 mg/kg) with a percentage

inhibition of 41.56%. This weak inhibition of ulcer index was accompanied by a significant reduction in the percentage of ulcerated surface (US). The US increased from 7.77% in the control group to 2.44 in rats treated with extract of *A. schweinfurthii* (500mg/kg). This reduction in the US was accompanied by a significant increase (p <0.05) in mucus production. The mucus weight increased from 52.81 ± 6.92 mg in the control group to 87.07±3.58 mg in rats treated with the extract (500 mg/kg). This significant reduction in the ulcerated surface shows that the extract of *A. schweinfurthii*, in addition to stimulating the secretion of mucus, inhibits the complex physiological mechanisms and oxidative stress induced by absolute ethanol. This efficiency is comparable to that of the methylene chloride extract of the leaves of *Bidens pilosa* on gastric ulcers induced by absolute ethanol in rats (Tan *et al.,* 2000).

The indomethacin method of inducing gastric lesions is a rapid and convenient way of screening plant extracts for anti-ulcer potency in macroscopically and microscopically visible lesions (Guth *et al.,* 1984). The pathogenic mode of action of indomethacin on the rat gastric mucosa involves the inhibition of cyclo-oxygenases 1 and 2 (Cox 1 and 2). This prevents the formation of prostaglandin PDH_2, which is the precursor for the production of all other PGs subtypes. PGs play important roles in many physiological processes. In the gastro-intestinal (GI) tract, PGs are very important mediators of mucosal defence and repair. Inhibition of their synthesis renders GI tissues much more susceptible to damage induced by luminal irritants (including gastric acid and pepsin), and less able to restore mucosal structure and function after injury. Suppression of PG synthesis is the key effect of indomethacin that leads to gastro-duodenal ulceration and bleeding (John, 2013). The present results demonstrate that the aqueous extract of *Anthocleista schweinfurthii* protects the rat gastric mucosa against hemorrhagic lesions produced by indomethacin. It is known that the curative action of some anti-ulcer drugs is mediated by the action of endogenous prostaglandins which promote mucus secretion and play an important role in the maintenance of the integrity of the gastric mucous layer against the actions of various damaging agents. The oral administration of *Anthocleista schweinfurthii* provoked a non significant increase in mucus production (Table 3). The action of sucralfate on mucus secretion was lower than that of the extract. It is known that sucralfate's main action is through the increase of mucus production by activating Cox 1 and 2 (Miller, 1982). So, the non significant increase showed by sucralfate confirmed the inhibitory action of indomethacin on cyclo-oxygenases. Consequently, we can suppose that *Anthocleista schweinfurthii* may increase mucus production *via* different pathways to ensure cytoprotection. Previous studies have shown that the extract of *Anthocleista schweinfurthii* directly stimulate the mucus secretion (Mezui *et al.,* 2015b). This is confirmed by the significant (P<0.01)

dose-dependent reduction in the ulcer index as well as the ulcerated surface. Previous studies showed that the aqueous extract of *Anthocleista schweinfurthii* significantly (P<0.01) induced mucus production during stomach ulceration caused by acetic acid (Mezui *et al.,* 2015b). This result may imply that the extract also acts through the prostaglandin pathway.

Direct necrolytic action of indomethacin on the gastric mucosa is more severe than that due to the reduction of gastric cytoprotective mechanisms induced by indomethacin. In addition to inhibiting the secretion of endogenous prostaglandins that results in the reduction of gastric blood flow, decreased secretion of bicarbonate, mucus and the surface phospholipids (Bommelaer and Tournut, 1989), indomethacin inactives gastric peroxidase thereby inducing tissue necrosis by reactive oxygen species (Chattopadhyay *et al.,* 2006). Non steroidal anti-inflammatory drugs (NSAIDs) were reported due to mitochondrial injury: to dissipate the mitochondrial transmembrane potential, and to mitochondrial permeability transition pore Induce, qui liberates cytochrome C. This enzyme generates reactive oxygen species (ROS) and thereby triggers caspase cellular lipid peroxidation waterfall, resulting and in cellular apoptosis (Nagano *et al.,* 2005). Indomethacin-induced gastropathy is mediated through generation of free radicals, neutrophil infiltration and disturbance in producing nitric oxide (Ihab, 2010).

Oxidative stress induced by indomethacin is manifested by an increase in lipid peroxidation in cell membranes and decreased thiol groups. These groups react readily with the active oxygen species. Indomethacin increases the rate of hydroxide radicals (OH⁻) and the inactivation of gastric mucosa peroxidase, which causes an increase in the endogenous hydrogen peroxide (H_2O_2) and its derived products (Chattopadhyay *et al.,* 2006). Indomethacin also reduces the activity of antioxidant enzymes such as catalase, superoxide dismutase and glutathione S-transferase (Mohamed *et al.,* 2014). The animals subjected to indomethacin presented a decrease in the quantities of SOD and catalase in the stomachal tissue compared with the normal rats (Table 4). Oral administration of *Anthocleista schweinfurthii* reduced the ulcer index very significantly (P<0.01). These results are similar to those obtained by (Kwiécien *et al* 2004) who showed that pentoxifilline possesses gastroprotective properties against stress-induced gastric damage, putting into evidence the antioxidative role of lipid peroxidation antioxidizing enzymes and proinflammatory cytokine. This increase in SOD and catalase in the gastric tissue might explain the reduction of ulcers because these compounds are endogenous antioxidants. SOD is capable of eliminating the superoxide anion by a reaction of dismutation, forming with two superoxides a molecule of oxygen and a molecule of hydrogen peroxide (dismutation) (Favier, 2003). Catalase eliminates excess hydrogen peroxide transforming it into a simple molecule of water

192

Mezui Christophe. *et al. / International Journal of Phytopharmacology.* 7(4), 2016, 184-195.

(Favier, 2003). MDA experienced a significant dose-dependent reduction in the rats treated with the extract of *Anthocleista schweinfurthii* at the tested doses. A reduction of the tissular concentration of MDA is proof of the reduction of oxidative stress (Kwiécien *et al.*, 2004) because MDA is a product of lipid peroxidation. The same results were obtained with leaves methanol extract of *Lantana camara* (Thamotharan *et al.,* 2010) and a flavonoid of *Citrus sinensis* (hesperidin) (Papiya and Kailash, 2014). The induction of gastric ulcer by indomethacin was evident from the structural abnormalities of the stomach mucosa. Histological analysis clearly demonstrated the alteration of mucosal structural integrity as revealed by its destruction in the negative control group (B) (Figure 2). This is due to the release of $^{.}OH^{-}$ radicals which trigger the shedding of stomach mucosa. The 500 mg/kg extract seem to act as a scavenger of OH^{-} and thus significantly prevents indomethacin-induced gastric mucosal apoptosis, and the associated gastric ulcer. Phytochemical studies of *A. schweinfurthii* have revealed the presence of flavonoids, polyphenols, tannins and leucoanthocyans (Njayou *et al.,* 2000). Tannins are known to protect the outermost layer of mucosa and to render it less permeable and more resistant to chemicals and mechanical injury or irritation and thus prevent ulcer development (Heloina *et al.,* 2008). Flavonoids and polyphenols protect the stomach against the oxidation and corrosion by trapping and neutralizing free radicals (Borrali and Izzo, 2000). The extract would protect the gastric mucosa *via* flavonoids and polyphenols. Flavonoids have anticarcinogenic activity. These are singlet oxygen scavengers and inhibitors of several lipoxygenase enzymes (Chung *et al.,* 1998). Flavonoids protect the gastric mucosal lesions by increasing the neutral glycoproteins, secretion of mucous and bicarbonate ions, by increasing endogenous prostaglandins, and by inhibiting the release of histamine from mastocyts cells (Zayachkivska, 2005).

The studies of acute and subacute toxicity showed that the extract of *Anthocleista schweinfurthii* is slightly toxic (Mezui *et al.,* 2015a). In the present study, we extended toxicity study specifically to study the possible toxic effects of the extract on the estrous cycle since the phytoestrogens have gastro-cytoprotective effects. Before the start of the experiment, the cycle of each animal was studied during two weeks, thus covering three complete estrous cycles. The purpose of this preliminary study was to verify, before the treatment, that the animals had a normal estrous cycle. During the experiment, the administration of three doses of extract (250mg/kg; 500mg/kg; 1000mg/kg) during 14 days, thus covering three complete cycles (OECD N0 415.1983) showed significant effect of the extract, neither on the alternation of the phases of the estrous cycle (Figure 2), nor over the duration of this cycle (Table 5). The vagina smears carried out made it possible to identify the various phases of the cycle. These phases were recognized by the presence epithelial keratinized cells (phase proestrus), of

the cornified cells (phase oestrus); cornified cells associated with polynuclear (phase metoestrus); the polynuclear alone (phase diestrus) (Figure 2). The presence of these four phases, in regular alternation, defines a complete estrous cycle (Byers *et al.,* 2012).

At the end of this study, it was disclosed that there was neither elongation nor a significant shortening of the cycle phases. It was also revealed that no influence of the extract, or the number of phases or on the order of succession of the different phases of the cycle occured. Vaginal cells showed no significant deformation. Indeed, substances that disrupt the estrous cycle work by lengthening or shortening the phases of the cycle, which may change or not the cycle (Moustapha *et al.,* 2011), or by causing deformation of vaginal cells (Hazarika and Sarma, 2007). These substances can also upset the order of succession of the different phases of the cycle. Sometimes certain phases of the cycle disappear or the estrous cycle can be locked to a specific phase (Dheeraj *et al.*, 2010). These different phases of the estrous cycle are regulated by ovarian hormones (estrogen and progesterone) secreted by the theca interna and granulosa mature follicles and the corpus luteum. Secretion of these ovarian hormones is stimulated by the pituitary gonadotropins (Prakash and Mathur, 1980). Keratinisation of the vaginal epithelium in adult female rats was caused by estrogen. The anti-estrogenic substances inhibit keratinization of the vaginal epithelium (Lerner, 1969). Furthermore, administration of an anti-estrogen to mature female rats leads to cycle arrest and decreased corneal cells in the vaginal smear (Dheeraj *et al*., 2010). Figure 2 and Table 5 show that the extract of *Anthocleista schweinfurthii* has no anti-estrogenic properties because the rats treated with the extract showed no disturbance of the estrous cycle compared to the batch control. The phytochemical study of the barks of *A. schweinfurthii* showed that they contain not only steroids but also alkaloids (Schmelzer and Gurib-Fakim, 2008). The alkaloids erythroidines being one of the classes of the phytœstrogenes (Djiogue *et al*., 2014), the barks of *A. schweinfurthii* would thus have estrogenic effects.

The uterus is one of the preferred targets of estrogens that prepare the implantation of a fertilized egg (Sherwood, 2006). The uterus reacts to cyclical variations of the levels of estrogen and progesterone by water retention and cell proliferation. These hormones cause an increase of uterine weight and size of the endometrium (Brien *et al.,* 2006). The aqueous extract of *A. schweinfurthii* led to a significant increase (p <0.05) in the relative weights of the uterus (Table 6). The increase in uterine weight is a basic index which characterizes a substance's estrogenicity after its effect on the uterine muscle (Shibeshi *et al.,* 2006). The aqueous extract of *A. schweinfurthii* induced a significant (p <0.01) and dose-dependent increase on the size of the uterine epithelium (endometrium) at all doses of the extract (Table 6 and Figure 3). This extract would have estrogenic properties because the estrogenic substances acting on the

uterus resulting in fluid retention and cell proliferation (Njamen *et al.,* 2014). Secondary metabolites in *A. schweinfurthii* could either increase the vascular permeability of the uterus and cause water infiltration into the uterus, or stimulate the synthesis of specific proteins having attached to the uterus receptors and initiate a cascade of genomic responses.

The aqueous extract of *A. schweinfurthii* induced a dose-dependent increase in the cholesterol level in ovarian tissue with a significant difference (p <0.01) at extract doses of 500 mg/kg and 1000 mg/kg (Table 6). This high rate of total cholesterol in the ovaries of female rats treated with the extract would suggest that the bark of *A. schweinfurthii* could contain phytoestrogens. Cholesterol is the precursor for the synthesis of steroid hormones, and the intake of exogenous estrogens promotes the cholesterol-aside in the ovaries. High levels of estrogen lead to a negative feedback at the pituitary gland which inhibits the secretion of ACTH and FSH (Marieb and Hoehn, 2010). Therefore, inhibition of steroidogenesis results in high total cholesterol in the ovaries. The extract of *A. schweinfurthii* therefore has estrogenic effects. This would justify the traditional use of the extract of the bark of *A. schweinfurthii* to treat ovarian disorders (Ruijter, 2007).

These estrogenic properties of the aqueous extract of the bark of *A. schweinfurthii* could explain the gastro-cytoprotective and curative effects of this extract on gastric ulcers (Mezui *et al.,* 2015b). Indeed, it is known that estrogens inhibit the secretion of gastric acid by decreasing the mass of the parietal cells of the stomach (Kayode, 1991). Ovariectomized rats treated with 17β-estradiol has

showed statistically significant reduction in gastric acid secretion induced with all the secretagogues (Amure and Omole, 1970). Additionally, the duodenal secretion of bicarbonate ions is significantly higher in women compared to men (20-29 years). On the other hand, the duodenal secretion of the ions bicarbonates does not present any significant difference at the men compared to the women (60 - 69 years) (Tuo *et al.,* 2011). Previous studies excluded a role of progesterone because gastrointestinal epithelia appear to lack the expression of progesterone receptors and progesterone even at higher doses did not induce bicarbonate secretion in mice (Tuo *et al,* 2011). The ability possessed by estrogenic compounds to reduce stomach acid and stimulate the secretion of bicarbonate ions explains their anti-ulcer effects.

CONCLUSION

The aqueous extract of the bark of *Anthocleista schweinfurthii* would possess anti-ulcerogenic effects due to its ability to strengthen the mucus barrier and its antioxidant properties. This extract would not have any harmful effect on the estrous cycle; it would rather have the estrogenic effects which would justify its anti-ulcerogenic effects and its traditional use in the treatment of the ovarian affections.

CONFLICT OF INTEREST:
The authors declare that they have no conflict of interest.

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