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Aqueous Extract of *Enantia chlorantha* (Annonaceae) Prevents the Delay in Chronic Gastric Ulcer Healing Caused by Indomethacin in Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors KTMM and TVP designed the study and wrote the protocol, authors KTMM and MC managed the biochemical analysis, author EOEG conducted the analysis and interpretation of histological sections, authors KTMM and TVP did the literature search and statistical analysis, author KTMM wrote the first draft, authors TVP and NZE supervised the study. Authors NZE and NB did the phytochemical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the ability of *Enantia chlorantha* aqueous extract to heal acetic acid-induced chronic gastric ulcers and to prevent the delay in chronic ulcer healing induced by indomethacin.
Study Design: Random allocation of male rats to groups of five rats each.
Place and Duration of Study: Department of Animal Biology and Physiology, Animal Physiology

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Laboratory (Gastroenterology Research Unit) University of Yaoundé 1 and Department of Biomedical Sciences (Pathology Unit), University of Buea between January and April 2015.
Methodology: Gastric ulcers were produced 5 days after submucosal injection of 30% acetic acid (0.05 ml) at the lesser curvature of rat stomachs corpus. The extract (250 and 500 mg/kg) was administered *p.o.* during 10 days. Ulcer healing was delayed by indomethacin administered *s.c.* at 1 mg/kg once daily for 2 weeks from 5 days after the acid injection. Extract or sucralfate were administered concomitantly. Mucus secretion and oxidative stress parameters (superoxide dismutase (SOD), malondialdehyde (MDA), reduced glutathione (GSH), catalase (CAT)) were measured, and macroscopic and histological assessment of ulcer healing was done.
Results: Ulcer healing rates were 82.7% and 88.6% for the 250 and 500 mg/kg doses of extract following 10 day treatment of acetic acid-induced ulcers *vs* 55.7% and 85.2% for spontaneous healing and Ranititine, respectively. Spontaneous healing (60.9%) was significantly (P<0.01) delayed by indomethacin (22.5% healing rate). Co-administration of extract (250-500 mg/kg) or sucralfate (100 mg/ kg) significantly (P<.001) inhibited the adverse effect of indomethacin, raising healing rates to 76.7%, 82.2% and 85.8%, respectively. Indomethacin significantly depressed gastric mucus production (39.79 mg) but extract and sucralfate restored values to 40.08-55.75 mg and 70.06 mg, respectively. Indomethacin raised MDA levels and decreased antioxidant enzyme levels. These effects were counteracted by *E. chlorantha* extract. Microscopy showed advanced re-epithelialisation with recovery of ulcer craters due to extract.
Conclusion: *E. chlorantha* accelerates the spontaneous healing of acetic acid-induced chronic gastric ulcers, and prevents the delay in chronic gastric ulcer healing caused by indomethacin. The healing-promoting effect of *the* extract could be due not only to stimulation of gastric mucus secretion but also to enhanced re-epithelialisation and inhibition of enhanced lipid peroxidation in the ulcerated gastric tissue.

Keywords: Enantia chlorantha; chronic gastric ulcers; delayed healing; antioxidant status.

1. INTRODUCTION

The introduction in 1969 of the type 1 acetic acid-induced chronic gastric ulcer model has greatly contributed to advances in the discovery of novel and effective antiulcer drugs [1,2]. Other workers later created three modifications of the methodology including the now famous (Type 2) model whose efficiency in antiulcer studies have been proven [3,4]. This model is termed chronic because the ulcers produced persist for a long time and resemble human chronic ulcers both grossly and histologically [1]. Clinical and experimental data indicate that traditional non steroidal anti-inflammatory drugs (NSAIDs) delay the healing of peptic ulcers by interfering with the action of growth factors, decreasing epithelial cell proliferation in the ulcer margin, decreasing angiogenesis in the ulcer bed, and slowing the maturation of the granulation tissue [5]. Other workers [6,7] reported that a 4-week course of indomethacin clearly delayed the spontaneous healing of acetic acid-induced ulcers in rats. The term “unhealed gastric ulcers” was coined to represent chronic ulcers that persisted for up to 12 weeks even after cessation of four week treatment with NSAIDs [8], and the mechanism underlying their production involves severe fibrosis, persistent neutrophil infiltration and poor angiogenesis at the ulcer base [9]. Increased polymorphonuclear cell infiltration had earlier been found to be the major histological abnormality persisting after cessation of indomethacin treatment [10], and significant enhancement of healing is promoted by anti-secretory drugs (e.g. omeprazole), prostaglandin analogs, mucosal defense agents (e.g. sucralfate), and various growth factors [4]. Mechanisms underlying delayed healing need to be well understood so that new therapies can be developed [11], and the increasing interest in traditional medicines has been attributed to the economic advantage they provide, the accessibility and assumed safety they offer when compared to conventional medicines [12,13].

Enantia chlorantha is an ornamental tree widely used in African pharmacopeia to treat many diseases. Quantitative and qualitative phytochemical screening of *E. chlorantha* aqueous extract showed a significant presence of alkaloids and phenolics, phytochemicals to which both the antimalarial [14] and the broad-spectrum antibacterial activities [15,16] have been attributed. Low doses of the aqueous extract of *E chlorantha* (50 mg/kg) have been shown to increase sperm motility and viability [17,18]. The antioxidant properties of various

solvent extracts of *E. chlorantha* have also been attributed to their flavonoid and phenolic contents [19]. The extract has antitusive, wound healing [20], antiviral [21] and hepatoprotective [22], activity, and HEPASOR® (Labothera Laboratories), a combination of protoberberine alkaloids (palmatine 65%, jatrorrhizine 20% and coloumbamine 15%) from *E. chlorantha* bark is sold for the treatment and prevention of viral hepatitis in Cameroon [23]. Previous work revealed anti *Helicobacter pylori*, prophylactic and healing properties of an anti-ulcer alkaloid (7, 8-dihydro-8-hydroxypalmatine) from *E. chlorantha* [24]. The acute and sub-acute toxicity profile of the aqueous stem bark extract of the plant has also been investigated [25]. The wide medicinal usefulness of *E. chlorantha* has led to an overexploitation of this plant and steps are being taken through macro propagation to prevent extinction [26]. Since the stem bark aqueous extract of *E. chlorantha* showed cytoprotective antioxidant properties in rats [27], we undertook the present study to evaluate the healing effects of the extract on both chronic acetic acid-induced gastric ulcers and on indomethacin delayed chronic gastric ulcers.

2. MATERIALS AND METHODS

2.1 Preparation of Plant Extract

The stem bark of *E. chlorantha* was harvested in Ambam, South region of Cameroon in July 2014. The plant was identified in comparison with the specimen n° 25918/SRFCAM held at the Cameroon National Herbarium. The fresh stem-bark of *E. chlorantha* was cut up, dried and ground to a fine powder. A mixture, 10% (w/v) of the powder and distilled water was boiled for 20 minutes, and then cooled to room temperature. After filtration of the decoction obtained through Wattman filter paper number 3, the filtrate was evaporated at 40°C using a *Raven* convection air oven (Jencons PLS, UK). The yellowish dried solid obtained (4.53%) was stored at 4°C and used later for our pharmacological tests.

2.2 Animals

Male Wistar albino rats (140-190 grams) raised in the animal house of the Faculty of Science, University of Yaounde I were used. The animals were fed a standard laboratory diet and given fresh water *ad libitum.* The authorization for the use of laboratory animals in this study was obtained from the Cameroun National Ethics committee (Reg. No FWA-IRB00001954).

2.3 Phytochemical Tests

Phytochemical tests for major metabolites of the extract were performed. The aqueous extract of *E. chlorantha* was screened for the presence of biologically active compounds such as tannins, alkaloids, saponins, flavonoids, anthocyanins, phenols, quinones, coumarins, sterols, triterpenoids, glycosides, proteins. Based on the intensity of coloration, the lather or the precipitate formed during the test, secondary metabolite proportions were characterized as present (++), weakly present (+), and absent (−) when the test result was negative.

2.4 Induction of Gastric Ulcers

2.4.1 Induction of simple chronic acetic acid ulcers

The glacial acetic acid chronic ulcer model described by [28] was used. Briefly, laparotomy was performed under ether anesthesia on experimental rats after a 24 h fast. Fifty microlitres of 30% glacial acetic acid were injected into the submucosal layer of the gastric wall of the stomach corpus at the region of the lesser curvature and the stomach wall wiped using cotton wool soaked in a 0.9% NaCl solution. The abdominal incisions were stitched up and feeding was resumed. Disinfectant (Betadine) was applied daily to the incised region to avoid infection. Four days after the operation, a control group of five rats (group1) was sacrificed under ether anesthesia, and the rat stomachs were opened in order to establish the degree of ulceration prior to the onset of treatment. Day 5 was then considered as the initial day of ulcer establishment. The remaining rats were divided into five groups of five rats each: group 2 (ulcerated negative control) received 1 mL of distilled water daily by gavage for 10 days, while groups 3 and 4 were given, respectively, 250, and 500 mg/kg of the extract. Group 5 rats were given 50 mg/kg of ranitidine (Azantac). An additional group of 5 healthy non ulcerated rats was included but the rats were given neither the extract nor ranitidine. On the final day the rats were sacrificed, and ulcer indices and gastric mucus production were measured. Ulcer healing rates were calculated by comparing the ulcer status of extract- and ranitidine-treated rats with those of the day 4 ulcerated controls. The degree of auto-healing was also evaluated by comparing the untreated controls given vehicle with the day 4 ulcerated controls. The stomach ulcerated portions were

fixed and stored in 10% formaldehyde awaiting histological studies.

2.4.2 Induction of hard healed chronic gastric ulcers

The same protocol described above was performed but with slight modifications in accordance with the procedure described by [6]. Following establishment of simple (Type 2) acetic acid-induced gastric ulcers, hard healed ulcers were produced by daily indomethacin treatment delivered (from day 5) as a subcutaneous dose (1 mg/kg) for 2 weeks. Indomethacin was suspended in saline solution and the vehicle alone was administered for the same period as a control at the volume of 1 ml/200 g BW. Another group of 5 ulcerated rats received only indomethacin daily for 2 weeks. The three remaining groups of ulcerated rats received the extract (250 and 500 mg/kg) or sucralfate concomitantly with indomethacin for 2 weeks. On the 15th day following ulcer induction, the animals were sacrificed using ether, and blood and gastric tissue samples were taken and prepared for the measurement of different oxidative stress parameters. Measurement of mucus production and macroscopic and histological study was done as described above.

2.5 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 [29].

2.6 Measurement of *In vivo* Antioxidant Capacity

Cellular glutathione (GSH) was measured based on the reaction between 2,2-dithio-5,5-dibenzoic acid and the thiol (SH) groups of glutathione to yield a complex whose absorbance was read at 412 nm [30]. The glutathione concentration was calculated using the molar extinction coefficient $\varepsilon = 1.36\ 10^4\ M^{-1}\ cm^{-1}$. Superoxide dismutase (SOD) activity was measured using a standard method [31] and expressed in U/mg of protein, while catalase was determined and expressed as mM of H_2O_2/min/mg of protein [32]. Tissue protein was measured using the Biuret method of protein assay. Lipid peroxidation was assessed by measuring the levels of malondialdehyde (MDA) [33]. Quantification of MDA was done using an extinction coefficient of $\varepsilon = 1.56\ 10^5\ M^{-1}\ cm^{-1}$.

2.7 Statistical Analysis

The data were analyzed using the one way analysis of variance (ANOVA) followed by the student-Newman- Keuls test. *P* values <.05 were considered significant. Values in tables are given as arithmetic means ± standard error of the mean (S.E.M.)

3. RESULTS

3.1 Phytochemical Screening

The preliminary phytochemical screening carried out on the aqueous extract of *E. chlorantha* revealed the presence of many phytoconstituents. These included tannins, saponins, anthocyanins, acids, glycosides (++), alkaloids, ketones, flavonoids, sugars, coumarins, amino-acids and proteins (+); phenols, quinines, oils, sterols, triterpenoids and resins (-) were not detected.

3.2 Ulcer Healing

The macroscopic aspect of the stomachs showed deep and wide craters in the control group (day 1 of ulceration) (Fig. 1B) representing an ulcerated area of 47.40±5.13 mm^2 (7,02% of glandular area) on the day of ulceration (Table1). Spontaneous healing (in control rats treated with vehicle for 10 days) reduced the ulcerated area to 21.00±4.53 mm^2 representing an auto healing rate of 55.7%. The extract of *E. chlorantha* (250 and 500 mg/kg) and ranitidine (50 mg/kg) significantly reduced ulcer craters after 10 days treatment to 8.20±1.11 mm^2, 5.40±0.73 mm^2 and 7.00±1.48 mm^2, representing healing rates of 82.7%, 88.6% and 85.2%, respectively. The macroscopic reductions in ulcer crater size were accompanied by significant increases in mucus production in the extract-treated and sucralfate-treated groups (56.1-75.6 mg) compared with the day 0 control (33.9 mg). Spontaneous healing was also accompanied by significant (*P*<0.01) mucus production (Table 1).

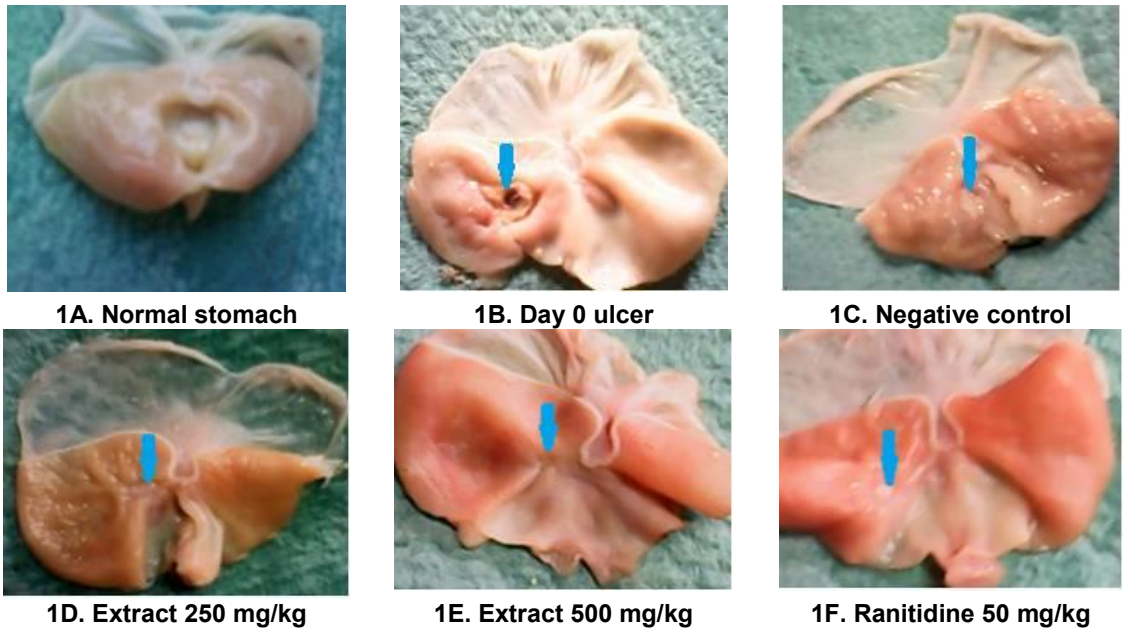
Glacial acetic acid was applied to rat stomachs on the serosal side and the rats were sacrificed 4 days after ulcer induction in order to ascertain the establishment of deep chronic ulcer craters. Stomach sections of these rats showed obvious loss of substance in the superficial mucosal layers. There was a diffuse mature infiltrate of mononuclear inflammatory cells, with the presence of congestion and edema which are

indicative of an acute ulceration. The muscular layers were spared (Fig. 2B & C). In the negative control rats that were maintained for 10 days after ulcer establishment but without anti-ulcer treatment, the entire glandular depths of the stomach sections were invaded by inflammatory cells. The superficial layers remained ulcerated, and the muscular layers were attained by the inflammatory process (Fig. 2D). Figs. 2E & F show different magnifications of ulcer sections from animals that received the plant extract at the dose of 250 mg/kg for 10 days. Although macromorphological pictures showed advanced topical healing, the histological sections still showed an invasion of the glandular depths by inflammatory cells. In the rats that received the extract at 500 mg/kg, the ulcer sections showed advanced signs of perfect healing, with granulation tissue and fibrosis (Figs. 2G & H). With ranitidine treatment for 10 days (Fig. I & J), the healing process was depicted by the re-establishment of mucosal loss. However, the inflammatory process could still be seen as depicted by intra muscular edema and sparse inflammatory cells.

Table 1. Healing effect of the aqueous extract of *E. chlorantha* on chronic acetic acid-induced gastric ulcers in rats

Treatment	Dose (mg/kg)	N	% ulcerated area	(%) healing	Mucus production (mg)
Control 1	-	5	7.02	-	33.91±2.34
Control 2	-	5	3.11	55.70	97.52±2.88**
E. chlorantha	250	5	1.21	82.70	56.10±4.64**
E. chlorantha	500	5	0.8	88.61	75.63±2.36***
Ranitidine	50	5	1.03	85.23	75.09±3.75***

Control 1 *(4 day ulcerated rats);* Control 2 *(spontaneous healing).**P<.01; ***P<.001; Statistically significant relative to Control 1*



1A. Normal stomach **1B. Day 0 ulcer** **1C. Negative control**

1D. Extract 250 mg/kg **1E. Extract 500 mg/kg** **1F. Ranitidine 50 mg/kg**

Fig. 1. Macroscopic aspect of simple chronic acetic acid-induced gastric ulcers

1A: Normal (Healthy control rat), 1B: Control 1 (ulcerated rat sacrificed 4 days after acetic acid ulcer induction), showing a deep large ulcer; 1C: Negative control; (ulcerated rat given vehicle for 10 days following ulcer induction). 1D &1E: ulcerated rats treated with 250 and 500 mg/kg of extract, respectively, for 10 days after ulcer induction. 1F: Positive control rat given ranitidine (50 mg/kg) for 10 days following ulcer induction. (Arrows indicate position of ulcer)

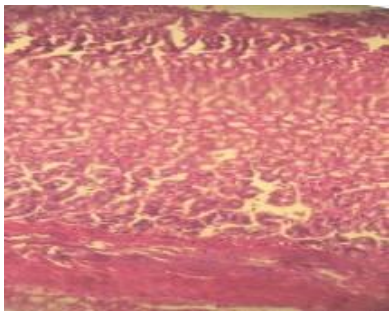


Fig. 2A. Normal rat stomach (x100)

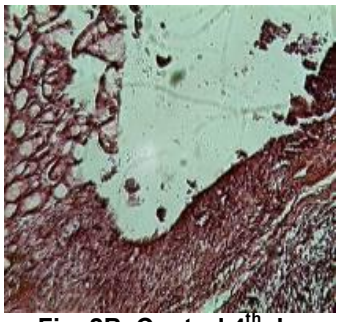
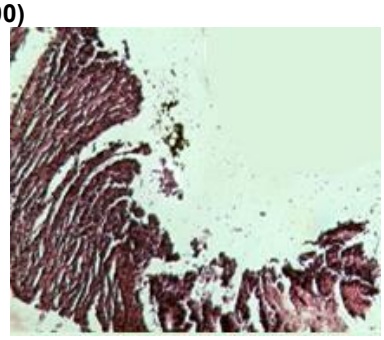
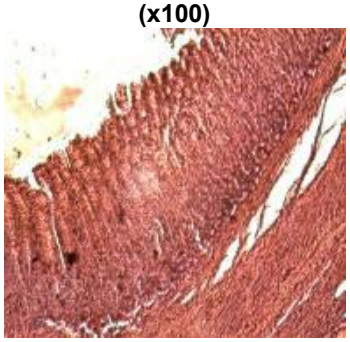


Fig. 2B. Control 4th day (x100)

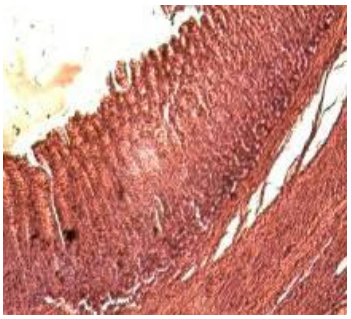
2C. Control 4th day (x100)



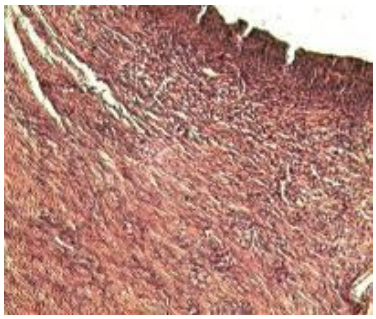
2D. Negative control (x100)



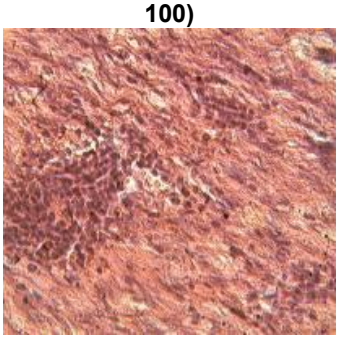
2E. Extract 250 mg/kg (x 100)



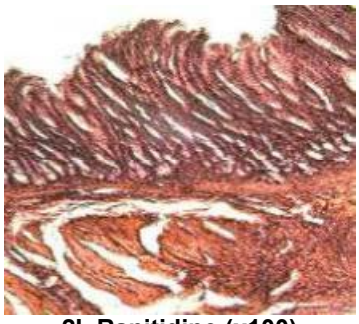
2F. Extract 250 mg/kg (x010)



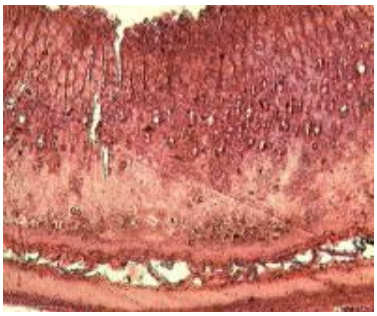
2G. Extract 500 mg/kg (x 100)



2H. Extract 500 mg/kg (x400)



2I. Ranitidine (x100)



2J. Ranitidine (x100)

Fig. 2. Histological presentation of acetic acid-induced chronic ulcers

2A: histological section of normal rat stomach showing gastric mucosa , intact annular muscles , and longitudinal muscles in the muscularis. 2B & C: Sections of control rats, 4 days after ulceration showing the depth of the ulcer with a superficial loss of substance and glandular destruction. 2D: Negative control (distilled water for 10 days) shows lymphocyte infiltration. 2E & F: Stomach sections of extract-treated rats (250 mg/kg) after 10-days treatment showing a recovering of the crater, and some edema (e). 2G & H: sections of extract-treated rats (500 mg/kg) after 10-day treatment, showing glandular proliferation and lymphocyte infiltration. 2I & J: Positive control rats treated with ranitidine showing healthy mucosa

The macroscopic aspects of the stomachs subjected to acetic acid ulcer induction followed by 2 week treatment with indomethacin are shown in Fig. 3. Compared with significant spontaneous healing that was observed in Fig. 3B, acetic ulcers treated with indomethacin for 2 weeks remained significantly sore and deep, without any visual signs of healing (Fig. 3C). These hard healed ulcers were on average 39.21±7.73 mm^2 in size (22.5% healing rate) compared with 50.6±6.72 mm^2 for the day 0 ulcer group. Treatment with extract and sucralfate significantly promoted the healing process (Fig. 3D, E, F). Thus, ulcerated areas reduced from 50.6±6.72 mm^2 on day 0 to 11.80±2.18 mm^2 and 9.00±2.19 mm^2 for 250 and 500 mg/kg doses, respectively, representing healing rates of 76.7 and 82.2%. Sucralfate promoted the highest reduction of ulcer index (85.8% healing rate) (Table 2). Significant increases in mucus production (*P*<.01) were observed in response to spontaneous healing, extract and sucralfate treatment.

The histological presentation of acetic acid-induced chronic ulcers in which healing was delayed by indomethacin treatment are shown in Fig. 4. In the rats sacrificed 4 days after ulcer induction in order to establish the initial chronic state of the ulcers, histological observation showed loss of mucosal substance, with congested blood vessels in the muscular layers and hemorrhagic foci in the lamina propria. The edema observed at all layers was consistent with a chronic ulcer (Fig. 4A). In the ulcerated rats that were allowed to heal spontaneously, ulcer sections showed foci of coagulative necrosis in the mucosa. The muscular layers showed congestion with regression of oedema. The presence of interstitial mononuclear cells in diffuse pattern attested to a chronic ulcer (Fig. 4B & C). When indomethacin was administered for 14 days to acetic acid ulcerated rats, the mucosal loss was accentuated, with more intense inflammatory infiltrate. There was no granulation tissue. This is a significant indication of the absence of healing. The presence of stromal fibrosis was evidence of a chronic non healing ulcer (Figs. 4D, E & F). When indomethacin treatment was accompanied by extract administration, the early stages of healing could be observed in response to the 250 mg/kg dose, the ulcers becoming shallow and limited to the lamina propria (Fig. 4 G&H). Treatment with the 500 mg/kg dose (Fig. 4I) and sucralfate (Fig. J & K) brought about full mucosal involvement by ulcer spanning the entire lamina propria and limited only by the muscularis mucosae. In addition, there was granulation tissue, testimony of healing.

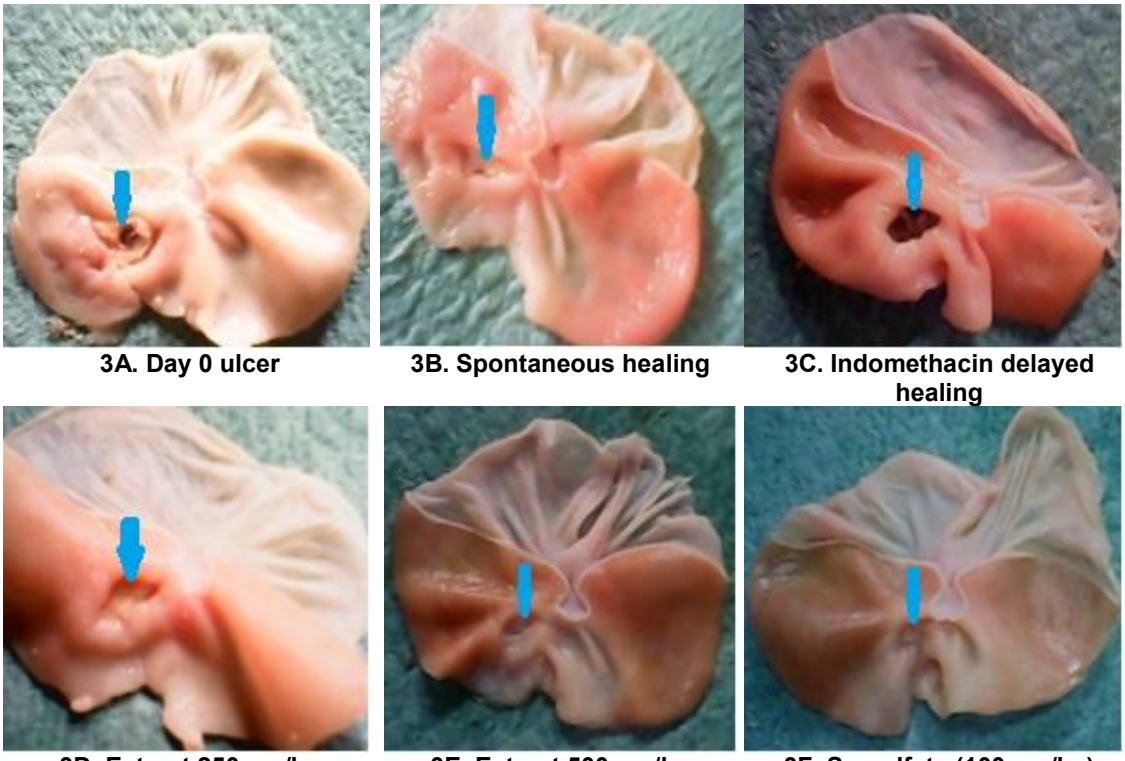
Table 3 shows the *in vivo* antioxidant effects of *E. chlorantha* extract in rats subjected to indomethacin treatment following acetic acid induction of chronic gastric ulcers. Results show that ulcer induction provoked a 51% increase in the blood plasma concentrations of MDA from 1.73±0.10 in normal rats to 2.62±0.19 µmol/ mg protein. The plasma MDA concentrations were further significantly (*P*=.05) raised to 2.71±0.14 µmol/ mg of protein on day 14 in rats with untreated hard healed ulcers, but concomitant extract administration (500 mg/kg) significantly (*P*<.001) reduced the MDA levels to (1.59±0.08 µmol/ mg protein) below normal values. The creation of hard healed ulcers reduced gastric

Table 2. Effect of the aqueous extract of *E. chlorantha* on indomethacin delayed healing of chronic acetic acid-induced gastric ulcers

Treatment	Dose (mg/kg)	N	% ulcerated area	Ulcer index	(%) Healing	Mucus production (mg)
Control 1	-	5	7.50	50.61±6.72	-	36.44±2.04
Control 2	-	5	2.93	19.80±2.91^{ee}	60.87	60.40±5.35^{ee}
Control 3	-	5	5.81	39.2±7.73**	22.53	39.79±1.23**
E. chlorantha	250	5	1.75	11.80±2.18$^{\Psi\Psi}$	76.68	46.08±1.76
E. chlorantha	500	5	1.37	9.00±2.19$^{\Psi\Psi\Psi}$	82. 21	55.75±4.77$^{\Psi\Psi}$
Sucralfate	100	5	1.07	7.20±1.63$^{\Psi\Psi\Psi}$	85.77	70.06±5.27$^{\Psi\Psi}$

*Control1: ulcerated rats killed 4 days post acetic acid injection, Control 2: Spontaneously healing ulcerated rats (without indomethacin) for 14 days, Control 3: ulcerated rats given indomethacin (Indomethacin delayed healing)14 days. N= numbers of rats.**P<.01; ***P<.001; Values statistically different relative to the control 2; ^{ee} P<.001; Values statistically different relative to the control 1; $^{\Psi\Psi}$ P<.01; $^{\Psi\Psi\Psi}$ P<.001;Statistically different relative to the control 3; The values are expressed as mean± SEM*

tissue antioxidant enzyme levels (GSH, catalase and SOD) by 94.9%, 32.4% and 67.5%, respectively, compared with normal non ulcerated rats. The extract of *E. chlorantha* raised the depressed antioxidant enzyme levels back towards normal values by 118%, 4.9% and 115%, for GSH, catalase and SOD, respectively, compared with the hard healed ulcer levels (Table 3).



3A. Day 0 ulcer **3B. Spontaneous healing** **3C. Indomethacin delayed healing**

3D. Extract 250 mg/kg **3E. Extract 500 mg/kg** **3F. Sucralfate (100 mg/kg)**

Fig. 3. Macroscopic aspect of rat stomachs subjected to indomethacin delayed healing on chronic acetic acid-induced gastric ulcers

3A: control 1 (ulcerated rats sacrificed 4 days after acetic acid ulcer induction), showing a deep large ulcer. 3B: control 2 (ulcerated rats given vehicle for 14 days following ulcer induction). 3C: control 3 (ulcerated rats given indomethacin for 14 days following ulcer induction). 3D & 3E: ulcerated rats treated with 250 and 500 mg/kg of extract, respectively, for 14 days after ulcer establishment. 3F: Positive control (rats given sucralfate (100 mg/kg) for 14 days following ulcer establishment). (Arrows indicate position of ulcer)

Table 3. Antioxidant effects of *Enantia chlorantha* extract in rats subjected to indomethacin treatment following acetic acid-induced chronic gastric ulcers

Treatment (mg/kg)	Dose (mg/kg)	MDA (µmol/ mg protein)	GSH (µmol/mg protein)	Catalase (mM H_2O_2/min/mg protein)	SOD (U/mg protein)
Normal rats	-	1.73±0.10	2.19±0.08	37.67±1.20	0.40±0.03
Control 1	-	2.62±0.19	0.11±0.01	32.01±0.94	0.31±0.04
Control 2	-	2.00±0.20	0.10±0.01	27.79±1.39	0.21±0.03
Control 3	-	2.71±0.14	0.11±0.01	25.45±0.15$^{\Psi}$	0.13±0.08
E. chlorantha	250	2.46±0.20	0.14±0.01	28.89±1.95	0.15±0.06
E. chlorantha	500	1.59±0.08***	0.24±0.01	26.70±0.93 $^{\Psi}$	0.28±0.05
sucralfate	100	1.85±0.08**	0.15±0.013	29.57±0.82	0.32±0.09

Control1: ulcerated rats killed 4 days post acetic acid injection, Control 2: Spontaneously healing ulcerated rats (without indomethacin) for 14 days, Control 3: ulcerated rats given indomethacin (Indomethacin delayed healing)14 days. N (numbers of rats) = 5.** P<.01; *** P<.001; Values statistically different relative to the control 3; $^{\Psi}$ P=.05; Statistically different relative to the control 1; The values are expressed as mean ± SEM

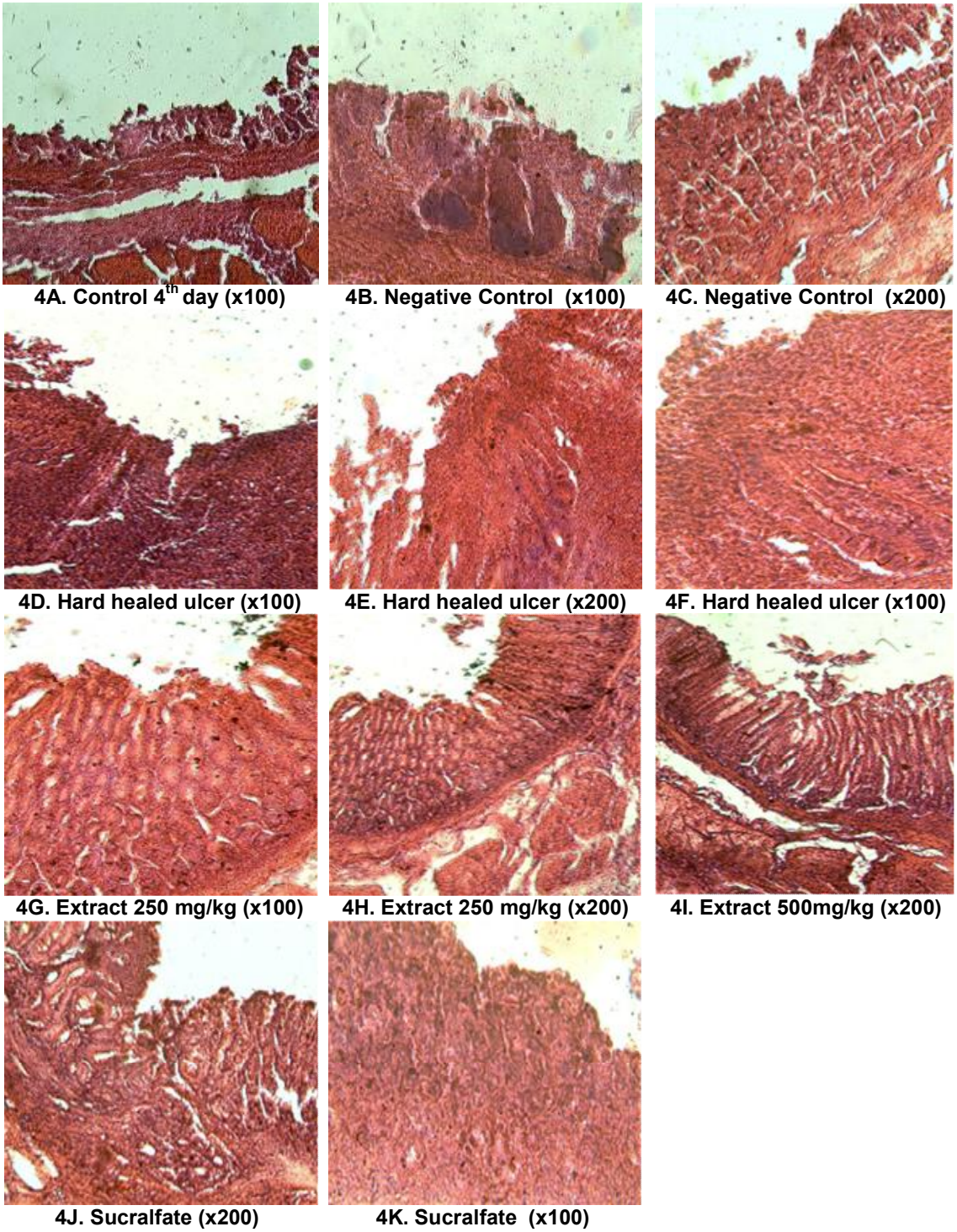


Fig. 4. Histological presentation of the hard healed chronic acetic acid-induced ulcers

4A; sections of control rats, 4 days after ulceration. 4B & C; Negative Control rats that had simple acetic acid ulcers, but with no Indomethacin for 14 days (spontaneous healing). 4D, E &F; Ulcerated rats that received indomethacin for 14 days (hard healed ulcers). 4G&H; Unhealed ulcer rats that received extract at 250 mg/kg. 4I; Unhealed Ulcer rats that received extract at 500 mg/kg. 4J & K; Positive Control Rats with chronic ulcer + Indomethacin + sucralfate treatment

4. DISCUSSION

The reported cytoprotective action and use of *E. chlorantha* bark in wound treatment [20,27] prompted our interest in the possible use for the treatment of hard healed ulcers. With the simple acetic acid-induced chronic gastric ulcers, the extract, administered *p.o*. once daily for 10 days, significantly accelerated the spontaneous healing rates of ulcers from 55.7% to 82.7% and 88.6% with the 250 mg/kg and 500 mg/kg doses, respectively. This acceleration of spontaneous healing was accompanied by significant promotion of mucus secretion. The importance of increased mucus strength in protecting the regenerating gastric epithelium is well-known [34, 35]. Healing is a normal physiological process that proceeds through a series of coordinated cellular events, culminating in the restoration of the functional integrity of tissues [11]. It can be observed on Fig. 2 that there was a decrease in areas of gastric mucosal necrosis, with glandular re-epithelialisation in treated groups, very marked with the 500 mg/kg extract dose. In the sucralfate-treated group, re-epithelialization was almost complete. Cellular proliferation plays an essential role in maintaining the integrity of the gastric mucosa [35]. Other workers observed that ulcer re-epithelialization is an essential process for gastrointestinal ulcer healing and, without restoration of a continuous epithelial barrier the mucosa would be vulnerable to mechanical or chemical injury and infections [36].

In the hard healed ulcer experiment, the extract also significantly prevented the delay in ulcer healing rate (22.2%) caused by indomethacin, the preventive rates being 76.7% and 82.2% with 250 mg/kg and 500 mg/kg, respectively. These preventive rates in the presence of indomethacin were remarkably similar compared with the healing rates (82-86%) obtained with the simple acetic acid ulcers. Sucralfate, at 100 mg/kg, had a higher preventive effect compared with the extract. Both steroidal and non-steroidal anti-inflammatory drugs negatively impact on the healing of experimental ulcers [1]. Repeatedly-administered indomethacin markedly prevents spontaneous healing of acetic acid-induced ulcers [9]. This is clearly demonstrated in Fig. 3C where indomethacin-induced delay of healing produced pronounced deep and wide ulcer craters which endured up to the 14^{th} day of treatment with no visible signs of spontaneous healing. Indomethacin treatment clearly affected the degree to which the extract and sucralfate promoted mucus production. On day 5 following ulcer induction histological observation showed diffuse mature infiltrates of mononuclear inflammatory cells, with the presence of congestion and edema which are indicative of an acute ulceration. In Spontaneous healing, the entire glandular depths of the stomach sections were invaded by inflammatory cells. The superficial layers remained ulcerated, and the muscular layers were attained by the inflammatory process (Fig. 2D). Severe fibrosis, persistent neutrophil infiltration, poor angiogenesis at the ulcer base, interference with the action of growth factors and slowing of the maturation of the granulation tissue are processes involved in the mechanism underlying the production of NSAID-induced hard healed ulcers [5,9], and increased polymorphonuclear cell infiltration is the major histological abnormality that persists after cessation of indomethacin treatment [10]. The advanced healing process initiated by sucralfate and *E. chlorantha* extract was evident from the significant presence of granulation tissue and the absence of fibrosis.

Indomethacin reduces prostaglandin secretion and gastric mucosal blood flow decreasing the natural healing of acetic acid-induced gastric ulcers [37]. The delayed healing can be prevented using exogenously-administered PGE_2 [6]. Thus the mechanism underlying indomethacin-induced delay of healing is related to the reduction in mucosal PGE_2 levels [9] that leads to a decrease in mucus production. When pretreatment with indomethacin reduces the cytoprotective efficiency of an antiulcer agent, this signals that mediation by endogenous PGs is involved. Unlike for *E. chlorantha* extract, this effect was not observed with sucralfate [27] which even at the dose of 1000 mg/kg did not affect the reduced PGE_2 content caused by indomethacin around chronic ulcers [38]. When a single dose of sucralfate dose-dependently and sustainably increased the volume and the pH of the gastric contents in a significant manner, the authors [38] suggested that the mechanism by which sucralfate accelerates the healing of gastric ulcers is related to its acid-neutralizing activity but not to endogenous PGs. The opposite effect is likely to be involved in the healing mechanism of action of *E. chlorantha* extract since it did not neutralize the highly acidic gastric medium created by pylorus ligation in rats [27]. Although it was concluded that only highly effective gastric acid inhibition reliably reverses NSAID-induced delay of gastric ulcer healing [5], *E. chlorantha* extract, with neither antisecretory

nor acid neutralizing potency [27], significantly prevented the indomethacin-induced delay of spontaneous healing in the present study. Other highly effective mechanisms, in addition to acid inhibition, must be involved.

Reactive unstable oxygen free radicals are believed to be generated during ischemia and are largely responsible for delayed healing due to their toxic effects on membrane lipid and proteins [39]. Oxidative stress resulting from the increased production of oxygen-derived free radicals (e.g. superoxide anion, hydrogen peroxide and hydroxyl radicals), has been known to take part in the pathogenesis of gastric ulcer [40]. Lipid peroxidation resulting from oxidative stress has been proposed to be the mechanism by which oxygen free radicals cause tissue damage [41]. Oxidative stress thereby causes cytotoxicity and delayed wound healing [42], and antioxidants help to protect cells from damage due to oxidative stress [43]. The in vitro antioxidant capacity of various solvent extracts of *E. chlorantha* has been demonstrated [19]. In this study, ulcer induction by acetic acid significantly raised the blood plasma concentrations of MDA from 1.73±0.10 in the non ulcerated controls to 2.62±0.19 µmol/ mg protein. Daily administration of indomethacin for 14 days further raised MDA concentrations to 2.71±0.14 µmol/mg protein, but concomitant extract administration significantly reduced the MDA levels to below normal values. In addition, *E. chlorantha* extract reversed the depression of gastric tissue antioxidant enzymes (GSH, catalase and SOD) caused by acetic acid and indomethacin, even though the values did not revert back to initial control levels. These results suggest that the ability of *E. chlorantha* extract to prevent the NSAID-induced delay of ulcer healing could be linked to its antioxidant (especially anti lipid peroxidation) activity. Plant products are potential agents for wound healing [44] and phytochemical screening of *E. chlorantha* extract revealed the presence of tannins, saponins, alkaloids and flavonoids, phytochemicals with known antiulcer activity [45-49].

5. CONCLUSION

The results of this study show that the aqueous extract of *E. chlorantha* accelerates the spontaneous healing of acetic acid-induced chronic gastric ulcers, and prevents the delay in chronic gastric ulcer healing caused by indomethacin. The healing-promoting effect of *E. chlorantha* extract in rats could be due not only to stimulation of gastric mucus secretion but also to enhanced re-epithelialisation and inhibition of enhanced lipid peroxidation in the ulcerated gastric tissue.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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