Mechanisms underlying the healing potentials of the methanol extract of *Chasmanthera dependens* stem on indomethacin-induced gastric ulcer

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**ABSTRACT**

*Chasmanthera dependens* stem is used in African traditional medicine as a remedy for various maladies; however, scientific evidence to validate its uses in gastric ulcer healing is lacking. This study investigated the mechanisms underlying the healing potentials of the methanol extract of *C. dependens* stem (MECD) on the indomethacin-induced gastric ulcer. Forty male Wistar rats at 8 weeks old were used in this study and divided into five groups \((n = 8)\). Group 1: control group, group 2: indomethacin alone (ulcerated untreated), group 3: ulcerated + 200 mg/kg MECD, group 4: ulcerated + 400 mg/kg MECD and group 5: ulcerated + 50 mg/kg cimetidine. Treatment with either MECD or cimetidine was once daily for 14 days after ulcer induction. Histological gastric injuries, antioxidant, inflammatory and apoptotic markers were evaluated. The results obtained revealed gastric mucosa damage as evident by marked histological changes, increased ulcer score, ulcer index, lipid peroxidation, Tumor necrosis-alpha (TNF-\(\alpha\)), Interleukin-1\(\beta\) (IL-1\(\beta\)), Cytochrome c (Cyt-c), Caspase-9 (Casp-9), Caspase-3 (Casp-3) and a number of apoptotic nuclei and significant decreased gastric antioxidant status, prostaglandin-E\(_2\) (PGE\(_2\)) and vascular endothelia growth factor (VEGF) levels at \(p < 0.01\). But MECD or cimetidine treatments significantly heal the ulcer and remarkably improved the biochemical parameters.

**Introduction**

Gastric ulcer is one of the leading chronic ailments of the gastrointestinal tract \([1,2]\) caused by administration of nonsteroidal anti-inflammatory drugs (NSAIDs), alcohol consumption, stress, *Helicobacter pylori*, smoking and nutritional deficiencies \([3]\). Untreated gastric ulcers may gradually deteriorate and result in unexpected complications, such as bleeding or perforation \([4,5]\). Indomethacin is a non-steroidal anti-inflammatory drug with antipyretic, antithrombotic and analgesic effects \([6]\). Its most common side effect is a gastrointestinal injury like gastric ulcers \([7]\). Mechanisms involved in indomethacin-induced gastric ulcer include inhibition of...
cyclooxygenase (COX), an enzyme responsible for prostaglandins synthesis, generation of free radicals, neutrophil infiltration, inhibition of angiogenesis and induction of apoptosis [8–12]. Prostaglandins are cytoprotective agents in the gastric mucosa, they stimulate the secretion of bicarbonate and mucus, maintain mucosal blood flow, and regulate mucosal cell turnover and repair [13]. Inhibition of prostaglandins synthesis, especially prostaglandin E2 (PGE2) by indomethacin and other members of the NSAIDs can slow down the healing process of gastric ulcers through their anti-inflammatory activity [14].

Although there is advancements in conventional chemistry and pharmacology in producing highly effective antiulcer drugs, like antacids, proton pump inhibitors, histamine receptor antagonists and anticholinergics, most of them have adverse side effects and are expensive [15,16]. Therefore, there is a need to develop drugs that will act as protective and remedial agents with potentially no side effects, effective and inexpensive for gastric ulcer therapy [17–19].

Medicinal plants have been used as substitute remedial agent in the management of gastric ulcers [20] and form part of unconventional medicine in developed and developing countries. Medicinal plants and their extracts play a crucial role against many diseases due to the presence of numerous phytochemicals found in them, their cost-effectiveness, availability and are with no significant side effects [21,22]. *Chasmanthera dependens* (Hochst) of the family Menispermaceae is a woody climber with a rough stem normally used in traditional medicine as a therapy for various diseases like red-eye infections [23], as a decoction for treating sexual related disorders, in the treatment of abdominal pain, sprained joints and bruises [23]. *C. dependens* possesses anti-inflammatory, analgesic and antifungal activity [24,25]. The plant contains berberine type alkaloids, palmatine, colombamine and jateorhizine, and some non-nitrogenous bitter principles such as chasmanthin, columbin and palmarin [26].

To date, there is paucity scientific evidence on the gastric ulcer healing potential of the stem of *C. dependens*. Therefore, this study evaluated the healing potentials of methanol extract *C. dependens* stem on indomethacin-induced gastric ulcer and the mechanisms involved in Wistar rats.

### Materials and methods

#### Animals and treatment

Forty adult male Wistar rats weighing 150.0–170 were purchased from the Central Animal House, Faculty of Basic Medical Science, College of Medicine, University of Ibadan, Nigeria and were kept in the Biochemistry Department animal house throughout the period of this experiment in polyethylene-walled cages. The rats were kept under standard laboratory conditions and were exposed to 12 h light and 12 h dark cycle at 25 ± 2°C. They were fed with standard rat’s chow (Ladokun Feeds, Nigeria) with water *ad libitum*. All procedures in this study conformed to the ‘Guide for the Care and Use of Laboratory Animals’ [27] published by the National Institute of Health (NIH publication revised, 1985) [28], and the study carried out according to the US NAS guidelines as approved by the University Ethical Committee. The rats were deprived of food for 24 hours before the administration of indomethacin, but allowed free access to clean water.

#### Plant material and preparation of Chasmanthera dependens extract

*C. dependens* stems were obtained from Iwo, Osun state, identified and authenticated by Mr. D.P.O. Esimekhua at the Department of
Botany of the University of Ibadan, Nigeria with voucher specimen (UIH22478) deposited at the herbarium. The stems were air dried and pulverized into coarse powders. 1.0 kg of the pulverized stem of *C. dependens* was subjected to maceration extraction using 5.0 liters of methanol with intermittent shaking for 72 hours. The mixture was filtered and the filtrate evaporated using a rotatory evaporator to obtain a semi solid mass of methanol extract of *C. dependens* (MECD). The semisolid extract was air dried and stored at −4°C for this study.

**Drugs and experimental protocol**

Cimetidine used for this study was manufactured by Greenfield Pharmaceutical Company Limited, Jiangsu, China while indomethacin was by Fabrique par: Yangzhou No. 3 Pharmaceutical Company Limited, Jiangsu, China and were obtained from a licensed pharmaceutical outlet in Ibadan, Oyo State, Nigeria. All other chemicals and reagents used were of analytical grade.

Rats were divided into five groups of eight rats each and were given indomethacin orally to induce gastric ulcer. Group 1 rats received 1% gum acacia solution. Groups 2–5 received 40 mg/kg body weight of indomethacin once. Twenty-four hours after gastric ulcer induction, rats in groups 3 and 4 were treated with 200 and 400 mg/kg body weight of MECDS and rats in group 5 were treated with 50 mg/kg body weight of cimetidine (CIM) orally for 14 days, respectively. Twenty-four hours after the last doses of the MECDS or CIM, all the rats were anesthetized with pentobarbital sodium (35 mg/kg, i.p.) and the blood was collected via the retro-orbital vein using a glass capillary tube from each rat. The blood was allowed to coagulate at room temperature and then centrifuged at 3000 rpm for 10 min to obtain serum. The serum was stored at −80°C for biochemical assays. Doses of the extract used in this study were based on our previous toxicity studies and the identification of tannin and flavonoids from the extract [29].

**Isolation of gastric mucosal tissue and assessment of gastric mucosal damage**

After blood collection, rats were sacrificed by cervical decapitation. The stomach of each rat was excised, rinsed immediately in ice-cold normal saline. Each stomach was opened along the greater curvature, rinsed and blotted dry and examined to locate the lesions using a 10× hand lens. Section of the stomach of each rat was cut and preserved for histological assessment and Tunnel assay. The degree of gastric mucosal damage was evaluated for gross pathology according to the method of Ohara et al. [30] based on the number and severity of gastric lesions. The severity of the gastric mucosal damage was graded as follows: no lesion = 0, hemorrhagic erosion (less than 5) = 1, hemorrhagic erosion (more than 5) = 2, many small linear ulcers (shorter than 2 mm) = 3, multiple linear ulcers of marked size, perforated ulcers = 5. The ulcer index (UI) for each group was calculated by multiplying the number of rats in each grade by the number of grades divided by the number of rats in each group lesions. Percentage healing was calculated by the following formula:

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\frac{(UI_{\text{IND control}} - UI_{\text{treated}})}{UI_{\text{IND control}}} \times 100.
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**Gastric mucosa tissue preparation**

After the assessment of gastric lesions, portions of the stomach from each rat were weighed and homogenized in ice-cold phosphate buffer saline (PBS) with Teflon homogenizer (Polytron, Heidolph RZR 1, Germany). The homogenates were centrifuged at a temperature of −4°C for 15 minutes at 10,000 revolutions per minute (rpm). The supernatant fraction for each rat was collected and stored at −80°C for estimations of antioxidant and oxidative parameters.
**Determination of gastric mucus content**

The gastric mucus content was estimated by the method of Corne et al. [31]. The concentration of Alcian blue was calculated from a calibration curve, and the results were expressed in μg/mg tissue.

**Estimation of antioxidant status and oxidative stress parameters**

Gastric mucosa superoxide dismutase (SOD) activity was determined by its ability to inhibit the auto-oxidation of epinephrine by the increase in absorbance at 480 nm as described by Sun and Zigma [32]. Catalase (CAT) activity in the gastric mucosa tissue supernatant was assessed according to the method of Evans and Diplock [33], GSH level was estimated according to the method of Beutler et al. [34]. Lipid peroxidation in the gastric tissue supernatant was determined by the method of Ruiz-Larrea et al. [35] by measuring the level of malondialdehyde (MDA), a stable product of lipid peroxidation and protein contents of the gastric mucosa supernatant was determined using the Lowry et al. method [36].

**Determination of serum TNF-α, IL-1β, PGE2 and VEGF levels**

The serum TNF-α, IL-1β and PGE2 levels were measured by commercial enzyme-linked immunosorbent assay (ELISA) using rat specific TNF-α, IL-1β and PGE2 assay kits (Cusabio Biotech Co., Ltd, Wuhan, China) following the manufacturer’s instructions. Vascular endothelia growth factor (VEGF) level (RayBiotech, Inc., USA) in the serum was measured by ELISA. All the experiments were performed in triplicates and the results were presented in pg/ml.

**Determination of serum Cyt c, Casp-9 and Casp-3 levels**

Serum Cyt c, CASP-9 and CASP-3 levels were measured by ELISA using rat specific Cyt c, CASP-9 and CASP-3 assay kits (Cusabio Biotech Co., Ltd, Wuhan, China) according to the manufacturer’s instructions. All the experiments were performed in triplicates and the results were presented in pg/ml.

**Terminal dUTP nick-end labeling (TUNEL) assay**

Paraffin-embedded sections of the gastric mucosa tissue of rats from each group were stained with the Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) technique using an in-situ apoptosis detection kit (Promega, USA) according to the manufacturer’s protocols and apoptotic cells were observed under a microscope and photographed.

**Histological assessment**

Histological assessment was performed according to the method of Laloo et al. [37]. Stomach sections from each rat of all the groups were fixed in 10% neutral buffered formalin, dehydrated in graded alcohol and embedded in paraffin. Fine (4–5 μm) sections were obtained, stained with hematoxylin-eosin (H & E) and slides were mounted for observation under a light microscope (Zeiss Jenaval, Jena, Germany) and photographed using a digital camera.

**Statistical analysis**

All data were expressed as mean ± standard error of the mean (SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Duncan’s multiple comparison test using SPSS software version 22 (SPSS Inc., Chicago, Illinois). Graphs were constructed using Graphpad Prism version 7.0. Values of p< 0.01 were considered statistically significant.
Results

Effect of MECD and CIM on ulcerogenic parameters

Oral administration of IND caused a significant ($p<0.01$) increase in ulcer score and UI with a decrease of the mucus content in the IND alone group rats compared to the control group (Figure 1), but post-treatment with either MECD or CIM at different doses for 14 days significantly decreased the ulcer score and UI and increased the mucus content as revealed by the percentage healing offered by MECD (78.48%, 84.74%) at 200 and 400 mg/kg body weight and CIM (80.34%) at 50 mg/kg body weight, respectively.

Effect of MECD and CIM treatment on antioxidant status and oxidative stress in IND-induced gastric ulcer healing

The effects of MECD and CIM on antioxidant status of the rat gastric mucosa were estimated and the results (Figure 2(a–c)) showed that the activities of SOD and CAT and the level of GSH were significantly ($p<0.01$)

Figure 1. Effect of methanol extract of Chasmanthera dependens stem on (a) ulcer score; (b) ulcer index and (c) mucus content in indomethacin-induced gastric ulcer healing in rats. Bars with different superscripts for each parameter are significantly different ($p<0.01$). a Significantly different from the control group ($p<0.01$); b Significantly different from the IND group ($p<0.01$). IND: indomethacin (40 mg/kg b. w.), MECD1: 200 mg/kg methanol extract of Chasmanthera dependens, MECD2: 400 mg/kg methanol extract of Chasmanthera dependens and CIM: 50 mg/kg cimetidine.
decreased in the IND alone group rats compared to the control rats. However, oral administration of MECD stem at 200 and 400 mg/kg significantly ($p < 0.01$) increased the activities of these enzymes and GSH level in a dose-dependent manner. The 50 mg/kg CIM group also revealed a significant ($p < 0.01$) increase in the activities of these enzymes and GSH level. Treatment with 400 mg/kg MECD stem reported values that comparable to the control group better than the standard drug. The effects of MECD or CIM on oxidative stress status of the gastric mucosa were assessed by measurement of the level of MDA, a biomarker of lipid peroxidation. IND significantly ($p < 0.01$) increased the level of MDA in IND alone group rats compared with the control group while post-treatments with MECD stem at 200 and 400 mg/kg or 50 mg/kg CIM significantly ($p < 0.01$) decreased the level of the MDA in the stomach when compared with the IND alone group rats stomach. Administration of 400 mg/kg MECD stem reduced the MDA level better than 50 mg/kg CIM (Figure 2(d)).
**Effect of MECD and CIM treatment on levels of TNF-α, IL-1β, PGE₂ and VEGF in IND-induced gastric ulcer healing**

Oral administration of IND increased the levels of TNF-α and IL-1β in the IND alone group significantly ($p < 0.01$) when compared with the control group. Post-treatment with 200 and 400 mg/kg of MECD stem significantly ($p < 0.01$) decreased the levels of both TNF-α and IL-1β when compared with the IND alone group and CIM also significantly ($p < 0.01$) decreased TNF-α and IL-1β levels when compared with the IND alone group (Figure 3(a,b)).

Figure 3(c,d) shows that oral administration of IND decreased the levels of PGE₂ and VEGF in IND alone group significantly ($p < 0.01$) when compared with the control group. Post-treatment with 200 and 400 mg/kg of MECD stem significantly ($p < 0.01$) increased the levels of both PGE₂ and VEGF when compared with the IND alone group and CIM also increased PGE₂ and VEGF levels significantly ($p < 0.01$) when compared with the IND alone group.

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**Figure 3.** Effect of methanol extract of *Chasmanthera dependens* stem on levels of (a) TNF-α; (b) IL-1β; (c) PGE₂ and (d) VEGF in indomethacin-induced gastric ulcer healing in rats. Bars with different superscripts for each parameter are significantly different ($p < 0.05$). aSignificantly different from the control group ($p < 0.05$); bsignificantly different from the IND group ($p < 0.05$). IND: indomethacin (40 mg/kg b. w.), MECD1: 200 mg/kg methanol extract of *Chasmanthera dependens*, MECD2: 400 mg/kg methanol extract of *Chasmanthera dependens* and CIM: 50 mg/kg cimetidine.
Effect of MECD and CIM on the levels of Cyt c, Casp-9 and Casp-3 in IND-induced gastric ulcer healing

We further examined the effect of MECD stem and CIM treatments on the gastric mucosa levels of Cyt c, Casp-9 and Casp-3. Our results showed that oral administration of IND significantly ($p<0.01$) increased the levels of Cyt c, Casp-9 and Casp-3 compared to the control group. Treatments with MECD stem or CIM decreased the levels of these intrinsic apoptotic markers. Again, the decrease at 400 mg/kg MECD stem was better than 50 mg/kg CIM and similar to the control group in all the markers (Figure 4(a–c)).

Histological assessment of the ulcer healing effect of MECD and CIM in IND-induced gastric ulcers

The stomach section of the control rats showed normal stomach architecture, the stomach sections of IND alone group showed an area of epithelial denudation marked with leukocytes infiltration while groups treated with 200 mg/kg and 400 mg/kg of MECD stem or 50 mg/kg
CIM showed regeneration, restitution and restoration of the surface epithelial cells and the entire gastric mucosal tissue with thickening of the submucosal (Figure 5(a–e)).
Effect of MECD on Terminal dUTP nick-end labeling (TUNEL) staining

In Situ analysis of TUNEL-positive gastric mucosal cells is shown in Figure 6. The IND alone group showed a high number of TUNEL positive nuclei (brownish color) when compared with the control group (Figure 6(b)). Post treatment with MECD or CIM decreased the number of TUNEL positive nuclei significantly when compared with the IND alone group (Figure 6(c–e)).
Discussion

Indomethacin-induced gastric ulcer is one of the most common methods for inducing gastric ulcer in experimental animals [13]. Indomethacin commonly delayed gastric ulcer healing via a continuous increase in gastric acid secretion, a decrease of mucus content and bicarbonate secretion and increased generation of reactive oxygen species (ROS) leading to lipid peroxidation [13]. The results of the present investigation revealed that oral administration of indomethacin to rats caused significant ulceration in the glandular region of the rat stomach as evident by histopathological assessment and a marked increase in ulcer score and UI. These results are consistent with previous studies [38] showing that indomethacin produces visible gastric ulcers in experimental animals. However, treatments with MECD stem or CIM decreased these ulcerogenic parameters. The decrease observed in the groups treated with MECD stem can be attributed to the synergistic effects of the phytocomponents present in the extract.

The gastric mucus is the first-line defense of the gastric mucosa against any aggressive ulcerogenic factors by providing significant buffering capacity for the neutralization of luminal acid [39]. Studies have also shown that gastric mucus has antioxidant activity that counteracts the gastric-ulceration mediated by oxygen free radicals and thus enhances the rate of local healing [40]. The decreased mucus content in the IND alone rats in this study, suggests the reduced protective ability of the mucosal membrane resulting in tissue damage. This result conformed to the previous report [41]. Again, the MECD stem elevated the mucus contents in the gastric mucosa, indicating enhanced mucus secretory potential of the MECD and its significant role in the ulcer healing process. CIM also increased the mucus contents in the gastric tissue but to a lesser extent.

Although indomethacin-induced gastric ulceration is usually attributed to the inhibition of prostaglandin synthesis, ROS have also been implicated in the pathogenesis and delayed gastric ulcer healing induced by IND in animal models [10]. The generated ROS can be counteracted by intracellular antioxidant systems, such as SOD, CAT and GSH [42]. In this study, the decrease in the SOD and CAT activities and GSH level with concomitant increase in MDA level in IND alone treated rats is consistent with the previous studies [41,43]. Furthermore, activities of SOD and CAT as well as the GSH level in rats treated with MECD stem were increased while the MDA level was reduced. These observations can be attributed to the presence of the phytocomponents that scavenge the ROS, particularly the flavonoids and the tannins that coat the mucosa and thus thicken the mucosal layer. Although CIM also produced a reversible effect of what was observed in the IND alone group, MECD at 400 mg/kg produced a better antioxidant capacity than CIM.

Infiltration of macrophages and neutrophils and the release of pro-inflammatory cytokines (such as TNF-α and IL-1β) significantly affect the healing and recurrence of the gastric ulcer [44]. TNF-α elicits its pro-inflammatory activity by increasing IL-1β production, induces cytotoxicity, apoptotic responses, leukocyte activation, tissue infiltration and suppresses gastric microcirculation around ulcerated mucosa and thus delays gastric ulcer healing [42,45,46]. The present investigation showed that administration of IND to rats increases the levels of TNF-α and IL-1β in the gastric tissue compared to the normal control group. This is in accordance with the previous report [47]. The significant (p) decrease in the levels of TNF-α and IL-1β in rats treated with the MECD stem suggests the capability of the extract to suppress inflammation and ROS generation. The reference drug, CIM, also decreased TNF-α and IL-1β levels significantly (p< 0.01).
The PGE₂ has been reported to play a crucial role in gastric ulcer healing by acting as an effective vasodilator, by increasing the release of mucus and bicarbonate and inhibiting gastric acid secretion [48,49]. Increased gastric PGE₂ level could downregulate inflammatory mediators, thus promoting gastric ulcer healing. In this study, the significant decrease in PGE₂ level in the IND alone group is in line with the previous study [45]. However, post-treatment with MECD stem increases the PGE₂, suggesting that the healing potential of MECD stem could be linked to the improved synthesis of functional PGE₂. Cimetidine also showed an appreciable enhancement of the PGE₂ level.

Growth factors like VEGF regulate angiogenic wound healing at its different stages [50,51]. The vascular endothelial growth factor is required to stimulate the formation of granulation tissue and the development of new microvessels. Thus, the decrease in the gastric VEGF in IND-treated rats in this study connotes a delay in the healing of the gastric ulcer induced by IND and this is in accordance with a previous report [51]. However, administration of MECD stem elevated the VEGF level in a dose-dependent manner, suggesting that MECD stem has angiogenic potential that stimulates vascularization resulting in healing of the gastric ulcer. The CIM also increases the VEGF significantly.

Apoptosis is a crucial process for maintaining cellular homeostasis and encompasses different cell proteases and response to various apoptotic signals. Cleavage and degradation of the proteases result in cell death. Activation of caspases has been documented as the key to the apoptotic process and triggers the cells to undergo apoptosis [52]. Mitochondrial apoptotic pathway is usually mediated by the release of cytochrome c resulting in a cascade of events that activate caspase-9 and subsequent activation of caspase-3, the downstream effector caspase. The increase in the levels of Cyt-c, CASP-9 and -3 in IND alone group in this present study may contribute to the delay in gastric ulcer healing by inducing apoptotic response via generation of ROS, altered transition potential and mitochondrial permeability [53] in the rats. This result is in accordance with the previous report [54]. Again, MECD stem and CIM reversed these increases significantly (p< 0.01). Furthermore, the antiapoptotic effect of MECD stem was verified by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay and the result showed that MECD reduced the number of the apoptotic nuclei in the treated groups better than cimetidine.

Conclusion

In conclusion, oral administration of methanol extract of C. dependens stem accelerates the healing of the indomethacin-induced gastric ulcer via enhancement of antioxidant status, the release of growth factors that induce angiogenesis like VEGF and PGE₂ and suppression of pro-inflammatory cytokines and intrinsic apoptotic pathway. The healing observed at 400 mg/kg MECD was better than that of cimetidine probably due to the synergistic effects of the phytoconstituents present in the MECD.

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