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Research Article

Bioassay-Guided Fractionation of the Crude Methanol Extract of *Cassia singueana* Leaves

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ABSTRACT

The crude methanol extract of *Cassia singueana* leaves was subjected to chromatographic separation using column and thin layer chromatographic techniques. The eluted fractions were screened for bioactivity using indomethacin-induced gastric ulcer model in rats. The extract yielded eight (8) component fractions. Fraction 8 (F8) exhibited the most profound anti-ulcer activity when it completely protected rat stomachs from experimentally-induced ulcerative effects caused by 40 mg/kg of oral indomethacin treatment. Further fractionation of F8 gave two second generation fractions (A and B). Sub-fraction 8_A or 8_B demonstrated a comparatively reduced *in vivo* anti-ulcer protective ability compared to the parent fraction (F8). The bioactive effects of the sub-fractions against indomethacin-induced ulcerogenic tendencies in rats appeared to be synergistic.

Keywords: Bioactivity, Bioassay, Column chromatography, Thin layer chromatography, Fractionation

1. INTRODUCTION

Peptic ulcer remains one disease in which multiple drug therapy is instituted for successful management. Drugs such as histamine H_2-receptor antagonists, proton pump inhibitors, analgesics, broad spectrum antibiotics, antacids and mucosal resistance enhancers are combined in its treatment [1]. This is because there is no known drug that proves solely effective in treating the disease. Persistent ulcer relapse compels patients to undertake intermittent or continuous therapy for many years [2]. The pathophysiology of gastric ulceration is due to an imbalance between aggressive factors (acid, pepsin, Helicobacter *pylori* and non-steroidal anti-inflammatory agents) and local mucosal defensive factors (mucous bicarbonate, blood flow and prostaglandins). Peptic ulcer results from over-bearing effects of the aggressive factors.

Fortunately, the plant kingdom has been discovered to provide alternative sources of chemotherapeutic agents. Plant plays important role in human life as the main source of food, medicine, wood, oxygen producer and many more [3]. A large section of the world's population relies on traditional remedies to treat plethora of diseases due to their low costs, easy access and reduced side effects [4]. In the present decade, there is rapid development in the field of medicinal chemistry research, yet many plant derived drugs cannot be synthetically produced. Some compounds e.g. atropine and reserpine are too expensive to be synthesized and the possibility of synthesizing many other useful drugs such as morphine, cocaine, ergometrin remains vague [5]. Isolation of plant derived drugs therefore holds relevance in drug discovery. The use of plants as medicine by human dates back to pre-historic times [6]. Many popular plant-derived drugs abound e.g. aspirin, an analgesic originated from *Salix alba* [7]; atropine, a parasympatholytic drug which could be used in the management of severe tetanus [8] is produced from *Atropa belladona*; quinine, an anti-malarial drug came from *Chinchona officinalis* and *Chinchona pubescens* bark [7]; morphine, a potent narcotic analgesic was produced from *Papaver sommniferum* [9]; vinblastine and vincristine anti-cancer drugs originated from *catharanthus roseus* [10]. Bioassay-guided fraction is a procedure whereby an extract is chromatographically fractionated and refractionated until a pure biologically active compound is isolated. Each fraction produced is evaluated in a bioassay system and only active fractions are further fractionated [11].

Cassia singueana leaves were used in ethnobotanical practice by the *Fulani* and *Hausa* tribes of Northern Nigeria to treat peptic ulcer cases. The methanol extract of the plant leaves demonstrated proven antiulcer activities in different gastric ulcer induction models [12-14] but there were no prior efforts at isolating the pure compounds responsible for the observed effects. The aim of the study was to isolate the anti-ulcer fraction(s) in the crude extract of *C. singueana* leaves.

2. MATERIAL AND METHODS

2.1. Chemicals, drugs, reagents and instruments

Freshly prepared solutions and analytical grade chemicals were used for the experiments. Concentrated tetraoxosulphate (VI) acid (H_2SO_4) from BDH laboratories, England; indomethacin, cimetidine (Sigma Aldrich, USA); methanol was obtained from Riedel-deHaen, Germany, hexane, chloroform, ethylacetate (Sigma Aldrich, USA), vanillin, atomizer, ultraviolet lamp (Yalien, China), beakers, test tubes and test tube racks, hot oven (Gallenkamp UK), analytical weighing balance (Metler), ceramic mortar and pestle, hot plate, graduated measuring cylinders, thin-layer chromatography (TLC) tank and TLC plates, silica gel for column and silica gel for TLC (Sigma Aldrich, USA) were used for the study.

2.2. Animals

Matured albino wistar rats of both sexes weighing 160-180 g, bred in the laboratory animal unit of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka were used for the study. The rats were kept in the same room with a temperature varying between 28 and 30^{0}C; lighting period was between 15 and 17 h daily. The rats were kept in stainless steel wire mesh cages which separated them from their faeces to prevent coprophagy. They were supplied clean drinking water and fed standard animal feed (Grower mash pellets, Vital feed®, Nigeria). The laboratory animals were used in accordance with laboratory practice regulation and the principle of laboratory animal care as documented by Zimmerman [15] and NIH [16].

2.3. Preparation and extraction of the plant material

Fresh leaves of *C. singueana* plant were collected and authenticated by a taxonomist from Botany department, University of Nigeria, Nsukka. The leaves were dried under mild sunlight and pulverized into powder in a grinding machine. Cold extraction was performed using 80% methanol for 48 h with intermittent shaking at 2 h interval. Removal of solvents *in vacuo* at 40^{0}C afforded 11.7% (w/w) of methanol extract. The extract was stored at 4^{0}C until used.

2.4. Fractionation of the crude extract of C. singueana leaves

2.4.1. Column chromatography

The crude extract of *C. singueana* (7 g) was subjected to column chromatography to separate the extract into its component fractions. Silica gel 60G was used as the stationary phase while varying solvent combinations of increasing polarity were used as the mobile phase [17]. In the setting up of column chromatography, the lower part of the glass column was stocked with glass wool with the aid of glass rod. The slurry prepared by mixing 150 g of silica gel and 350 ml of hexane was poured down carefully into the column. The tap of the glass column was left open to allow free flow of solvent into a conical flask below. The set-up was seen to be in order when the solvent drained freely without carrying either the silica gel or glass wool into the tap. At the end of the packing process, the tap was locked. The column was allowed 24 h to stabilize, after which, the clear solvent on top of the silica gel was allowed to drain down to the silica gel meniscus. The wet packing method was used in preparing the silica gel column. The sample was prepared in a ceramic mortar by adsorbing 8.0 g of the extract to 20 g of silica gel 60G in methanol and dried on a hot plate. Spatula was used to stir the sample continuously to dryness while guarding against getting it burnt. The dry powder was allowed to cool and then gently layered on top of the column. The column tap was opened to allow the eluent to flow at the rate of 40 drops per minute. Elution of the extract was done with solvent systems of gradually increasing polarity using hexane, chloroform, ethylacetate and methanol. The following ratios of solvent combinations were sequentially used in the elution process; Hexane: chloroform 100:0, 80:20, 60:40, 40: 60, and 20: 80; chloroform: ethylacetate 100:0, 80:20, 60:40, 40: 60, and 20: 80; ethylacetate: methanol 100:0, 80:20, 60:40, 40: 60, 20: 80 and 0:100. A measured volume (500 ml) of each solvent combination was collected gradually with a 10 ml syringe and sprayed uniformly by the sides of the glass into the column each time. This measure prevented solvent droplets from falling directly and disturbing the topmost layer of the column. Distortion of this layer would result in non-uniform drain of the fractions. The eluted fractions were collected in aliquots of 10 ml in test tubes.

2.4.2. Analytical thin layer chromatography (TLC) and pooling of fractions

The content of each test tube was spotted on pre-coated (silica gel F254) aluminium plates in a small chromatographic tank to separate the different fractions based on their relative mobilities in solvent systems and colour reactions with ultra-violet light. The fractions were concentrated in a rotovaporator at 40^{0}C, and 210 milibar. The mass of the different fractions was determined. Analytical TLC used precoated silica gel (GF254 on polyester plate). A strip of the precoated silica gel was cut out. With 1.0λ micro pipette, a spot of the sample was applied on the plate about 1.0 cm from the edge. It was dried using hot air dryer. The strip was lowered into a small chromatographic jar containing the solvent system. The jar was covered with a glass lid. The solvent was allowed to ascend until the solvent front was about ¾ of the length of the strip. The strip was removed and dried by a hot air dryer and viewed under UV lamp at 365 and 254 nm to identify the fluorescing spot. The fluorescent spot was marked and then sprayed with spray reagent: 0.16 g vanillin in 30 ml concentrated tetraoxosulphate (IV) acid (H_2SO4). The

strip was placed in hot oven at 110^{0}C for 5 seconds for visibility of fluorescent bands. The colour reaction was recorded and the relative Retention factor (Rf) value was calculated based on the formula described by Stahl [18]:

$$Rf = \frac{\text{Distance traveled by the streak from the starting point}}{\text{Distance traveled by the solvent from the starting point to the solvent front.}}$$

The fractions were kept at 4^{0}C in the refrigerator for further work.

2.5. In vivo bioassay screening of the fractions of C. singueana extract using indomethacin-induced gastric ulcer model in rats

A total of 27 albino wister rats of both sexes (150-185 g) were marked, weighed and assigned to 9 groups (A-I) of 3 rats per group. All the rats were fasted for 24 h prior to commencement of the experiment. Group A (negative control) was given only distilled water (10 ml/kg) orally, group B (positive control) received cimetidine (100 mg/kg, *per os*), while groups C, D, E, F, G, H, I and J were given oral treatments with 100 µg/kg of the corresponding fraction (F). Group C was given F1, group D received F2, group E was drenched with F3, group F with F4, group G with F5, group H with F6, group I with F7 and group J with F8 of the extract respectively. After 30 min, each rat was given an oral dose of indomethacin (40 mg/kg, *p.o.*). All treatments were administered through gastric intubation. The animals were then allowed 6 h before they were humanely sacrificed by cervical dislocation. The rat stomachs were carefully removed and cut open through the greater curvature with a scapel blade. After rinsing with distilled water, each stomach was pinned to a white background on a wooden board and examined for ulcer lesions with the aid of a magnifying lens (x10). Gross photographs of the gastric ulcers were taken for documentation.

2.6. Further bioassay-guided fractionation with Preparative TLC and screening of the fractions using indomethacin-induced gastric ulcer model in rats

Preparation of plates was done as described by Stahl [18] with modification. Thirty-five grammes of silica gel GF254 (Kieselgel 60 G Merck) was mixed with 85 ml of distilled water in a ceramic mortar and pestle to form slurry. The slurry was poured into the trough of a moveable spreader which was adjusted to 0.5 mm. The slurry was spread in a single passage onto five glass plates (20 x 20 cm) placed on an improvised aligning tray. Prior to the spreading of the slurry, the surfaces of the clean glass plates were made grease-free by cleaning them with methanol soaked in cotton wool. The freshly coated chromatographic plates were left on the tray until the transparency of the layer disappeared. The plates were subsequently activated for use in an oven for 1 h at 110^{0}C. The coated surface was marked (using a dissecting pin) on the straight edge. A 7 mm margin on both sides of the plate was marked and the area from the edge of the plate to the mark was not streaked. In the application of fractions, dropping pipettes (10 ʎ) were used to apply the various pooled fractions on the activated plates. Drops of the pooled fractions were applied in line to form a straight line streak or band. Each streak was dried before another one was superimposed on it. The streaked plates were run in a chromatographic tank containing 40 ml Chloroform-Ethylacetate-Methanol, 2:2:1 as the eluting solvent. The chromatographic tank was filled to the 0.5 cm mark with the eluting solvent. A white blotting paper was placed on each side of the tank to saturate the tank evenly with the vapour of the eluting solvent. The tank was immediately covered after introducing the eluting solvent and the white blotting papers. A smear of vaseline was applied to the edges of the underside of the coverlid of the tank to make sure that the lid fitted tightly to avoid escape of the vapour. The streaked chromatographic plates were put into the tank, two plates at a time at an angle of 30^0 from the edge of the tank. The eluting solvent was allowed to run for a distance of 15 cm starting from the streaked end, after which they were removed from the tank and allowed to dry. The same procedure was carried out for all other plates that were subsequently prepared and for all the pooled fractions. Each plate containing a single pooled fraction was viewed under ultra-violet lamp at 254 and 365 nm in a dark room. Separated zones were marked and the Rf values were calculated with the formula previously described.

Elution of separated spot zones: the marked separated zones were scrapped off the glass plates with a spatula onto a clean sheet of paper. The scrapings were transferred into centrifuge tubes containing 5 ml of absolute methanol. The content of the centrifuge tube was shaken manually for 10 mins. The eluent was separated from the adsorbent by centrifuging at 2500 rpm for 10 mins. This process was repeated until it was satisfactory that all the eluent was collected as much as possible. The collected eluent in methanol was evaporated to dryness using a hot air oven at the temperature of 40 ^{0}C. The eluents were subjected to further bioassay test to confirm biological activity using indomethacin-induced gastric ulcer model as described before in 2.5. Eluents that exhibited maximal *in vivo* stomach ulcer protective ability were then labeled as the active fractions.

3. RESULTS

3.1. Fractionation of the crude extract of C. singueana leaves

A total of eight (8) preliminary fractions of *C. singueana* extract were eluted and identified. The fluorescent characteristics of the fractions including their Rf values and wavelengths are presented in Table 1.

Table 1: Characteristics of fractions from the extract of Cassia singueana leaves

Fractions	Quantity (mg)	Spots (RF)	TLC coloration UV 254 nm	356 nm
F1	1.14	0.667	grey	Absorbed
F2	0.19	0.400	yellowish brown	Absorbed
F3	0.59	0.511	pink	Absorbed
F4	0.28	0.410	light yellow	Absorbed
F5	0.91	0.267	brown	Absorbed
F6	1.24	0.622	orange	Absorbed
F7	0.87	0.429	reddish brown	Absorbed
F8	0.211	0.824	dark brown	Absorbed

Eluent: chloroform - ethylacetate - methanol (3:2:1)

3.2. In vivo bioassay screening of the fractions of C. singueana extract using indomethacin-induced gastric ulcer model in rats

Group A (negative control): The rats were given only distilled water prior to gastric ulcer induction with indomethacin (40 mg/kg, *p.o.*). There were severe ulcerations in the gastric mucosa (Plate 1).

Group B (positive control): The rats received cimetidine (100 mg/kg, *p.o.*), a reference antiulcer drug, before stomach ulcers were induced with indomethacin (40 mg/kg, *p.o.*). The stomach epithelia of the rats were visibly protected and only a few (2 in number) focal ulcer lesions could be seen in one of the stomachs (Plate 2).

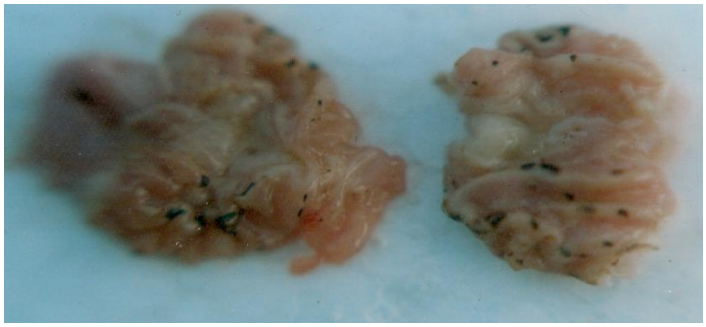


Plate 1: Gross photograph of rat stomachs from negative control showing deep and severe ulcer lesions in indomethacin (40 mg/kg) ulcer induction.

Groups C-I: Rats in the separate groups were given oral treatment (100 µg/kg) of one out of the first seven fractions (F_1-F_7) of *C. singueana* extract respectively, before gastric ulcer induction with indomethacin (40 mg/kg, *p.o.*). Severe gastric ulcer lesions comparable to the ones in the negative control rats (Plate 1) were produced.

Group J: Each rat in this group was given 100 µg/kg of the last fraction (F8) of the extract prior to gastric ulcer induction with indomethacin treatment. The rat stomachs were completely protected from ulceration (Plate 3).

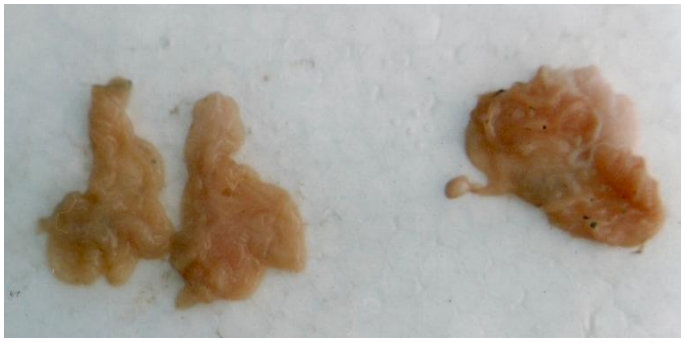


Plate 2: Gross appearance of cimetidine-protected rat stomachs with minimal focal ulcer lesions from indomethacin ulcer induction.

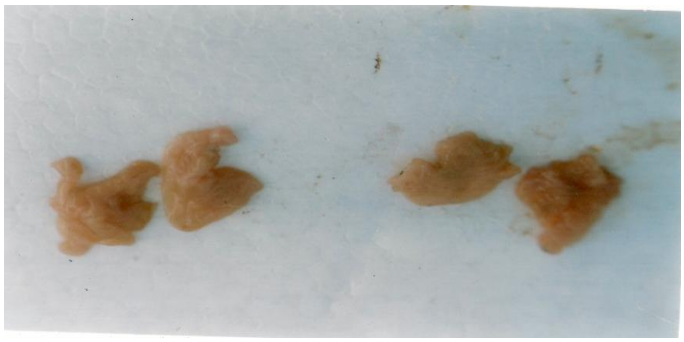
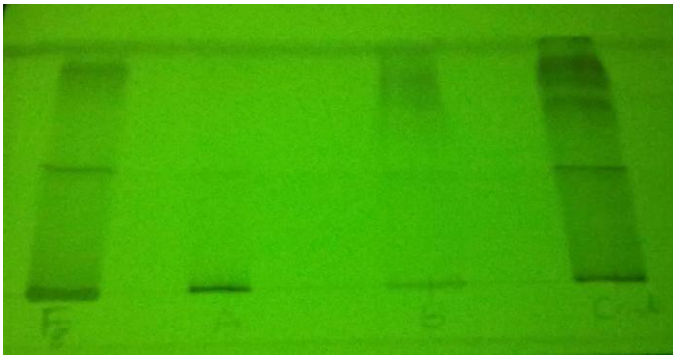


Plate 3: Stomachs from rats treated with F_8 showing complete protection against the ulcerative effects of indomethacin (40 mg/kg).

3.3. Further bioassay-guided fractionation with Preparative TLC and screening of the fractions using indomethacin-induced gastric ulcer model in rats

Fraction 8 (F8) became the most bioactive component of C. singueana extract against indomethacin-induced gastric ulceration in rats and further fractionation of it using TLC yielded two sub-fractions, A and B (Plate 4). Sub-fraction 8_A had Rf value of 0.235 while that of sub-fraction 8_B was 0.824. Further i*n vivo* tests with the sub-fractions (8_A and 8_B) using indomethacin-induced gastric model revealed that each sub-

fraction had a reduced protective ability against the ulcerative tendencies of indomethacin treatment compared to the parent fraction (F8).



F8 8_A 8_B Crude

Eluent: chloroform and methanol (2:2)

4. DISCUSSION

The crude extract of *C. singueana* leaves yielded 8 distinct fractions on column chromatography. Fraction 8 became the most bioactive component of the extract when it completely protected rat stomachs from experimentally-induced ulcerative lesions caused by 40 mg/kg of indomethacin treatment. Indomethacin, a non-steroidal anti-inflammatory drug (NSAID), inhibits cyclooxygenase conversion of arachidonic acid from cell membrane phospholipids to prostaglandins and thromboxanes. Prostaglandins induce inflammation but they are also needed for maintenance of the integrity of gastro-intestinal (GI) epithelium. NSAIDs cause prostaglandin cyto-protective deficiency which contributes to the pathogenesis of gastric injuries [19]. Cyclooxygenase I (COX-I) is a constitutive enzyme and its secretions are valuable for tissue homeostasis; its inhibition gives rise to GI ulceration [20]. Cyclooxygenase II (COX-2) expression on the other hand, is triggered off by an inflammatory process; hence selective inhibition of COX-2 represents selective inhibition of prostaglandin biosynthesis during inflammation [9]. NSAIDs including indomethacin suppress both COX-I and COX-II resulting in stomach ulcers. The effect of indomethacin on COX-1 enzyme was most likely blocked by the extract but the mechanism is not yet fully understood. Minimal ulcer lesions were produced in the stomach of rats that were pre-treated with 100 mg/kg of cimetidine, a popular anti-ulcer drug but there was no ulceration in the stomach of fraction 8-treated rats (Plate 3). This showed that F8 was more potent against indomethacin-induced ulcerogenesis compared to cimetidine. Further fractionation of F8 gave two sub-fractions (8_A and 8_B) which had reduced gastro-protective effects against toxic injury caused by indomethacin in the experimental rats. The anti-ulcer effects of sub-fractions 8_A and 8_B could therefore be synergistic. Bioassay-guided fractionation is a procedure whereby an extract is chromatographically fractionated and refractionated until pure biologically active compound is isolated. Bioactive compound can then be isolated through further preparative TLC. TLC is a basic method suitable for rapid detection of drug substances. It is a method for separating individual components comprising a sample by using thin layer of silica gel as a stationary phase [18]. TLC technique is advantageous in that it is selective, specific and rapid in identifying drug substances than the simple characterisation methods using chemical reagents which reveal substances by colour and precipitation reaction tests [21]. Chemical tests should however, complement TLC findings. There is no interference by excipients in TLC and the method can be used for identification, purity test and semi-quantitative estimation of the active ingredient in the dosage forms [22]. Each fraction produced was evaluated in an *in vivo* bioassay system using rats to facilitate selection of the bioactive fraction. The bioactive fraction would usually produce the desired biological activity which in this case, is inhibition of indomethacin-induced gastric ulceration in the experimental rats. Degradation or transformation of the active plant constituent may occur during fractionation due to hydrolysis, esterification, oxygenation or ultraviolet irradiation [21]. Again, decrease in activity after fractionation may be as a result of the phenomenon of synergy between the active ingredients.

5. CONCLUSION

Fraction 8 with Rf 0.824 from the crude methanol extract of *C. singueana* leaves was isolated as the most profound bioactive anti-ulcer agent in the plant leaves. It exhibited greater potency compared to cimetidine in experimentally-induced toxic assault from indomethacin in rats.

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