**Role of LTB4 and nitric oxide in the gastroprotective effect of *Prosthechea karwinskii* leaves extract in the indomethacin-induced gastric injury in the rat**

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## Abstract

Gastric injury is mainly described by inflammation of the gastric epithelium. Recently, our group of work demonstrated that *Prosthechea karwinskii* leaves extract induces both an *in vitro* antioxidative action, and *in vivo* gastroprotective effect in the rat. However, the molecules involved in the gastroprotective action by *Prosthechea karwinskii* are not known. Thus, the aim of this study was to determine whether *Prosthechea karwinskii* extract modifies anti-inflammatory and antioxidative biomarkers in an *in vivo rat* model of indomethacin-induced gastric injury. Rats were orally administered with indomethacin and *Prosthechea karwinskii* leaves extract. Our results suggest that the gastroprotective effect of *Prosthechea karwinskii* leaves extract is related to the

reduction of leukocyte infiltration and antioxidative action in a model of indomethacin-induced gastric injury. Further studies are warranted to investigate the role of the compounds identified in the gastroprotective action of *Prosthechea karwinskii* leaves extract.

## *Supplementary material*

### *Drugs and reagents*

Indomethacin (I7373) and omeprazole (O104) were obtained from Sigma-Aldrich (Toluca, México). Indomethacin was dissolved in 5% $NaHCO_3$, and omeprazole was dissolved in 0.9% saline solution. All reagents were prepared prior to use.

### *Plant material*

The collection, processing and extraction of *Prosthechea karwinskii* came from the same origin and is fully described by Barragán-Zárate et al., (2020). Specimens of *Prosthechea karwinskii* were collected in April 2016 from the ornaments used for Easter celebrations in the community of Villa de Zaachila (16°57' latitude N, 96°45' longitude W, 1490 m elev.), Oaxaca, with the permission of the organizers of this festival. A voucher specimen (Solano 4037) was identified and deposited in OAX Herbarium of the Instituto Politécnico Nacional. Only the leaves of these specimens were used, which were ground and sieved. Thereafter, the compounds were extracted.

Ultrasonic-assisted extraction was performed on a 750 W ultrasonic processor (VCX 750, Scientific SENNA), with a frequency of 20 KHz and an amplitude of 30%. The solvent employed was a mixture of ethanol/ water (50%; v/v). Ten grams of the powdered vegetal sample was mixed with 180 mL of solvent (mixture of ethanol/ water (50%; v/v)). Extraction was carried out at a temperature of 40°C during 20 min, with a pulse duration of 5-s on and 5-s off (Barragán-Zarate et al., 2020). *Prosthechea karwinskii* extract was dissolved in 0.9 % of saline solution with tween 20 for later use in the gastric damage model.

### *Animals*

Female Wistar rats, weighing 200-250 g, were obtained from Centro de Investigación y de Estudios Avanzados (CINVESTAV) del Instituto Politécnico Nacional (Mexico City, Mexico). All treatments for the animals, their care, and surgical procedures were

performed in accordance with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) with Bioethics Committee of ENMyH-IPN (registry number: ENMH- CBE/021/2019) and were in compliance with international rules and standards on the care and use of laboratory animals. Sample size per group consisted of six animals. Animals were fed standard laboratory chow and tap water *ad libitum*. Rats were placed in cages with wire-net floors to minimize coprophagy and fasted 12 h prior to experimentation but were allowed free access to tap water while fasting.

*Induction of gastric ulceration and assessment of gastric mucosal lesions*

Rats were randomly divided into equal groups and treated via oral gavage as follows: rats received oral indomethacin (30 mg/kg, p.o.) to induce gastric injury, 60 or 30 minutes before indomethacin, animals were administered with hydroethanolic extract of *Prosthechea karwinskii* (30, 100 and 300 mg/kg, p.o.) or omeprazole (30 mg/kg, p.o.), respectively. Vehicles of indomethacin, hydroethanolic extract of *Prosthechea karwinskii* and omeprazole were administered to control groups. Three hours after oral administration of indomethacin or the same volume of vehicle (5% $NaHCO_3$), rats were euthanized in a $CO_2$ chamber. Stomachs were removed, opened along the greater curvature, and thoroughly rinsed with saline solution (Pineda-Peña et al., 2018). The extent of the gastric-damaged area was scored blindly. For this, a picture of the fully extended stomach was taken; the length and width of each lesion was measured using ImageJ software (Version 1.45), and the total lesion area of the stomach ($mm^2$) was obtained for each rat. Based on the dose-response curve performed, we selected 300 mg/kg, p.o. of *Prosthechea karwinskii* extract further analysis. Samples of the corpus of the stomach were kept for further analysis.

*Histological study*

For histological assessment, gastric tissue was excised and fixed with 10% formaldehyde in phosphate buffered saline (PBS) for 24 h. These tissues were then washed with tap water, dehydrated in alcohol, and embedded in paraffin. Sections of 4-5 mm-thick were mounted on glass slides covered with silane. Haematoxylin and eosin staining was performed on each slide (Reyes-Gordillo et al., 2007), and slides were then examined under an optical microscope (Nikon Eclipse Slog) equipped with a high-resolution digital camera (Nikon Digital Sight DS-2mv).

*Determination of gastric mucosal $LTB_4$* and *NO*

A sample of the corpus region of the stomach was excised, weighed, and added to a tube containing 1 ml of PBS (10 mmol/l; pH 7.4). The tissue sample was minced with scissor for 30 s and then placed in a shaking water bath (37 °C) for 20 min. The samples were centrifuged (9000 g) for 1 min, and the supernatant was snap-frozen and then stored at -70 °C. The supernatant was used for determination of leukotriene B4 ($LTB_4$) levels by enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA kits from Cayman Chemical Co and Thermo Fisher Scientific, Inc. (Waltham, MA, USA), according to the manufacturer´s instructions (Pineda-Peña et al., 2018). NO levels were determined by nitrate/nitrite colorimetric assay using the Griess at 540 nm. The results are expressed as mmol/g of tissue (Díaz-Triste et al., 2014).

*Assessment of gastric mucosa SOD activity*

Samples were prepared by homogenizing gastric tissues on cold phosphate buffered saline solution and centrifuged at 900 g for 5 min at 4 °C. The resulting supernatant was used for superoxide dismutase (SOD) assay. SOD activity was determined using the method described by Sun et al. (1988) with minor modifications. The SOD value was determined by measuring the absorbance at 560 nm. SOD activity was calculated as $Umg^{-1}$ protein.

*Compound identification through UPLC-ESI-qTOF-MS/MS*

Compound identification was carried out using a UPLC liquid chromatography system (Ultimate 3000, Thermo Scientific) coupled to an Impact II mass spectrometer (Bruker) with electrospray ionization (ESI), method used is fully described by Barragan-Zarate et al., (2020). Tentative identification of compounds based on their spectral data (accurate mass and fragmentation patter) was obtain comparing MetaboBase Bruker's and Massbank's libraries.

*Statistical analysis*

All data are expressed as mean ± standard error of the mean (S.E.M.). Comparisons among controls were performed utilizing one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. $P \leq 0.05$ was considered as a statistically significant difference between means.

## Figure legends

Fig S1. Gastroprotective effect of *Prosthechea karwinskii* leaves extract (30, 100 and 300 mg/kg, p. o.) on the indomethacin-induced gastric injury model in rats. Data are represented as mean ± S.E.M. (n=6) * $P \leq 0.05$ versus respective basal, # $P \leq 0.05$ versus respective vehicle (Veh).

Fig S2. Representative histopathological sections of gastric mucosa following different treatments. A) Basal, B) *P. karwinskii* extract (300 mg/kg; p.o.) + 5% $NaHCO_3$, C) Indomethacin (IND; 30 mg/kg, p.o.) + saline solution (0.9%), D) *P. karwinskii* extract (300 mg/kg; p.o.) + IND, E) Omeprazole (30 mg/kg; p.o.) + IND.

Fig S3. Representative images of gastric lesions in the corpus of the stomach of different treatments. A) Basal, B) *P. karwinskii* extract (300 mg/kg; p.o.) + 5% $NaHCO_3$, C) Indomethacin (IND; 30 mg/kg, p.o.) + saline solution (0.9%), D) *P. karwinskii* (300 mg/kg mg/kg, p.o.) + IND, E) Omeprazole (30 mg/kg; p.o.) + IND.

Fig S4. UPLC-ESI-qTOF-MS/MS chromatogram of the Prosthechea karwinskii leaf ultrasonic extract. The following compounds were identified: 1 (Quinic acid), 2 (Malic acid), 3 (Neochlorogenic acid), 4 (Chlorogenic acid`), 5 (Rutin), 6 (Kaempferol diglucoside), 7 (Azealic acid), 8 (Pinellic acid), 9 (5-o-methyl embelin), 10 (Embelin).

## References

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Reyes-Gordillo K, Segovia J, Shibayama M, Vergara P, Moreno MG, Muriel P. 2007. Curcumin protects against acute liver damage in the rat by inhibiting NF-κB, proinflammatory cytokines production and oxidative stress. Biochim Biophys Acta-Gen Subj. 1770(6):989–996.

**Figure S1**

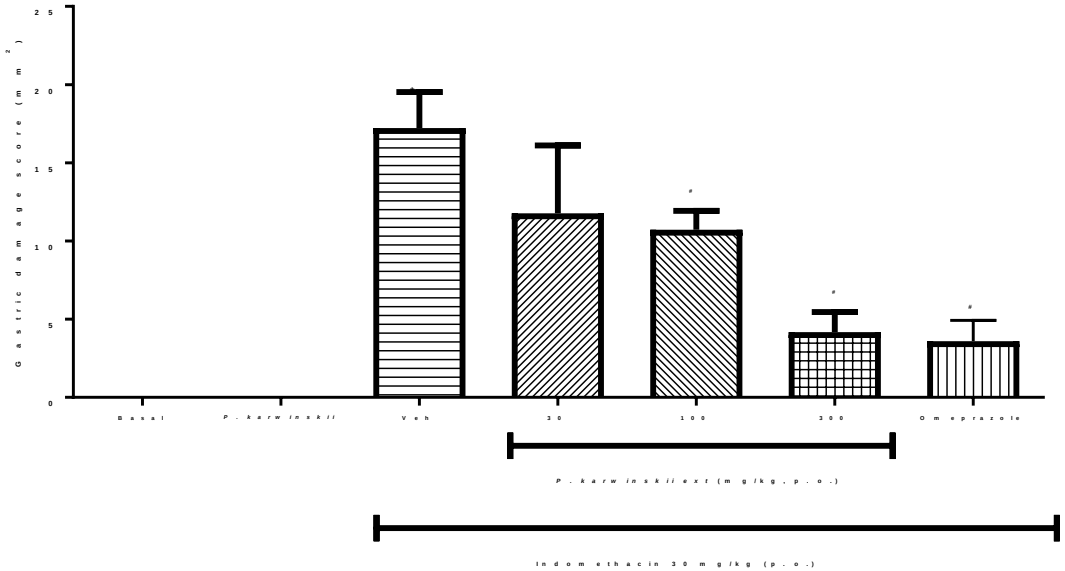
Gastric damage score (mm²)
25
20
15
10
5
0
Basal
P. karwinskii
Veh
30
100
300
Omeprazole
P. karwinskii ext (mg/kg, p.o.)
Indomethacin 30 mg/kg (p.o.)

**Figure S2**

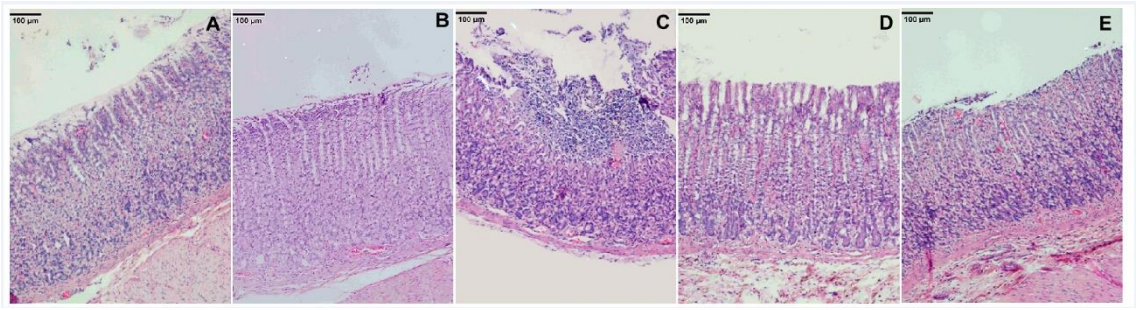

**Figure S3**

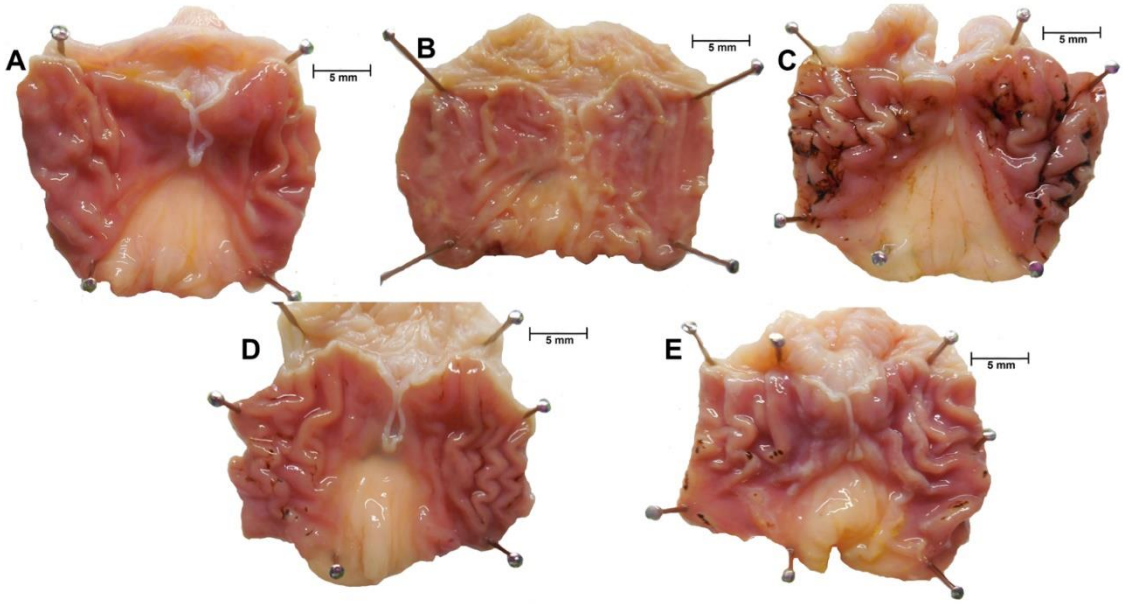

**Figure S4**

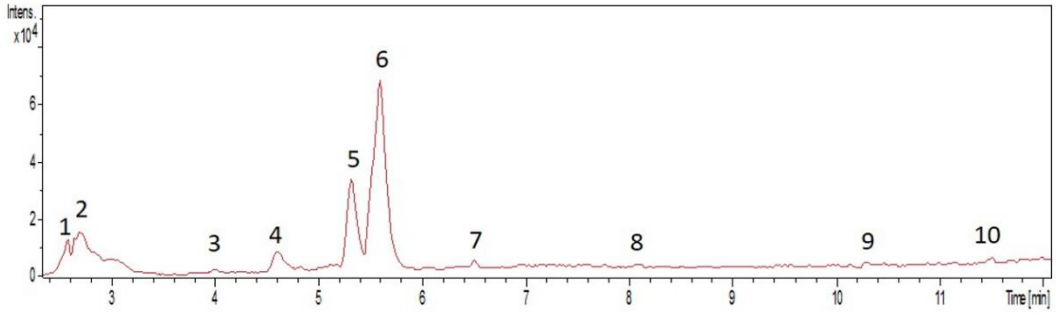

**Table S1**.Effect of hydroethanolic extract of *Prosthechea karwinskii* in the indomethacin-induced gastric damage in Wistar rats.

| Group/treatment | $LTB_4$ (pg/g tissue) | NO (mmol/mg tissue) | SOD (U/ng protein) |
|---|---|---|---|
| **Basal** | 210.6 ± 9.8 | 37.37 ± 2.5 | 0.28 ± 0.04 |
| **P. karwinskii + $NaHCO_3$ 5%** | 213.2 ± 24.4 | 47.6 ± 4.5 | 1.3 ± 0.09[b] |
| **Vehicle + indomethacin** | 265.0 ± 14.2[b] | 61.3 ± 4.9[b] | 0.5 ± 0.04 |
| **P. karwinskii + indomethacin** | 179.3 ± 13.2[a] | 45.1 ± 1.6[a] | 0.62 ± 0.1[b] |

Veh = Tween 20 + indomethacin (30 mg/kg, p.o.), P. karwinskii= hydroethanolic extract of *Prosthechea karwinskii* (300 mg/kg, p.o.). Values expressed as mean±S.E.M. (n = 5–7), statistical analysis was performed using ANOVA followed by Newman – Keuls test.

a $P<0.05$ vs. vehicle + indomethacin.

b $P<0.05$ vs. Basal

**Table S2**. Compounds identified by UPLC-ESI-qTOF in the *Prosthechea karwinskii* leaves ultrasonic extract

| Peak number | Compound | Type of compound | Retention time (min) | M/Z [M-1] | Chemical formula |
|---|---|---|---|---|---|
| **1** | Quinic acid | Carboxilic acid | 2.6 | 191.0569 | $C_7H_{11}O_6$ |
| **2** | Malic Acid | Dicarboxylic acid | 2.7 | 133.0142 | $C_4H_6O_5$ |
| **3** | Neochlorogenic acid | caffeoyl-quinic acid derivative | 4.0 | 353.0867 | $C_{16}H_{18}O_9$ |
| **4** | Chlorogenic acid´ | Caffeoyl-quinic acid derivative | 4.6 | 353.0867 | $C_{16}H_{18}O_9$ |
| **5** | Rutin | Flavonoid glycoside | 5.3 | 609.1464 | $C_{27}H_{29}O_{16}$ |
| **6** | Kaempferol diglucoside | Flavonoidl glycoside | 5.6 | 593.1518 | $C_{27}H_{29}O_{15}$ |
| **7** | Azealic acid | Dicarboxylic acid | 6.5 | 187.0997 | $C_9H_{15}O_4$ |
| **8** | Pinellic acid | Trihydroxyoctadecenoic acid | 8.1 | 329.2363 | $C_{18}H_{34}O_5$ |
| **9** | 5-o-methyl embelin | Monohydroxy-1,4-benzoquinones | 10.3 | 307.1937 | $C_{18}H_{28}O_4$ |
| **10** | Embelin | Benzoquinone | 11.5 | 293.1787 | $C_{17}H_{26}O_4$ |