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Study of the chloroformic extract of *Gnaphalium sp* in a model of alloxan-induced diabetes in Wistar rats. An alternative use of the "gordolobo" plant within traditional Mexican medicine

[Estudio del extracto clorofórmico de *Gnaphalium sp* en un modelo de diabetes inducida por aloxana en ratas Wistar. Una alternativa del uso de la planta "gordolobo" dentro de la medicina tradicional mexicana]

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Abstract: "Gordolobo" (*Gnaphalium sp*) is a Mexican medicinal plant understudied for the treatment of diabetes; therefore, the aim was to evaluate the chloroformic extract of G sp. (CEG) in alloxan-induced diabetic rats. Sesquiterpene lactones, polyphenolic compounds, triterpenes and steroids, apigenin, and lauric and myristic acid were identified in CEG by phytochemical, HPLC and GC-MS analysis; and the antioxidant capacity evaluated by FRAP, DPPH and ABTS, inhibited the formation of free radicals. There was no lethality or toxicity at doses of 2000 mg/kg. At doses of 200 mg/kg it did not decrease hyperglycemia; however, it did decrease biomarkers of oxidative stress (malondialdehyde, oxidized proteins, superoxide dismutase) associated with diabetes in pancreas. The β-cell function, insulin resistance and insulin sensitivity were not improved. In conclusion, CEG showed no hypoglycemic activity, but antioxidant activity in pancreatic tissue.

Keywords: *Gnaphalium sp*; Diabetes; Oxidative stress; Mullein; Polyphenolic compounds.

Resumen: "Gordolobo" (*Gnaphalium sp*) es una planta medicinal mexicana poco estudiada para el tratamiento de la diabetes; por el cual, el objetivo fue evaluar el extracto clorofórmico de G sp. (CEG) en ratas diabéticas inducidas por aloxana. Lactonas sesquiterpénicas, compuestos polifenólicos, triterpenos y esteroides, apigenina, y ácido láurico y mirístico, fueron identificados en CEG por análisis fitoquímico, HPLC y GC-MS; y, la capacidad antioxidante evaluada por FRAP, DPPH y ABTS, inhibió la formación de radicales libres. No hubo letalidad o toxicidad a dosis de 2000 mg/kg. A dosis de 200 mg/kg no disminuyó la hiperglucemia; sin embargo, si disminuyó los biomarcadores de estrés oxidativo (malondialdehído, proteínas oxidadas, superóxido dismutasa) asociados a la diabetes en páncreas. La función de las células β, la resistencia a la insulina y la sensibilidad a la misma no se vieron mejoradas. En conclusión, CEG no mostró actividad hipoglucemiante, pero si actividad antioxidante en tejido pancreático.

Palabras clave: *Gnaphalium sp*; Diabetes; Estrés oxidativo; Gordolobo; Extracto clorofórmico

INTRODUCTION

Diabetes mellitus is a metabolic disease that has affected 536.5 million people between 29 and 79 years of age worldwide (Sun *et al*., 2022). In Mexico, 13.4 million people affected are 58.95% women and 41.05% men, but the population aged 40 to 60 years (43.28%) and over 60 years of age (40.31%) has the highest incidence (Shamah-Levy *et al*., 2022). Given its high impact on Mexican population, diabetes mellitus is the second leading cause of death, behind heart disease and ahead of malignant tumors, closely associated with other conditions and comorbidities such as metabolic syndrome, and associated to oxidative stress tissular damage (INEGI, 2023).

Diabetes mellitus is a chronic metabolic disease characterized by Hiyoshi *et al*. (2019), associated with the development of physiological complications such as retinopathy, nephropathy, and diabetic neuropathy (Tomic *et al*., 2022), all of them have an important factor in common, which is oxidative stress, complications that are the origin of the cause of death in people with untreated diabetes.

Epidemiology has classified diabetes into 4 types (Genuth *et al*., 2018): type I, II, gestational, and MODY (maturity-onset diabetes of youth). Type I manifests as an autoimmune response that damages the β-cells of the pancreas, whereas type II is characterized by a failure of the signaling cascade in insulin-dependent tissues for blood glucose uptake, which is known as insulin resistance (Redondo *et al*., 2020). Both types of diabetes are of high incidence worldwide (Casamitjana & Oriola, 2004; McIntyre *et al*., 2019).

Existing pharmacological strategies encompass different mechanisms of action against diabetes, some of the types of drugs used are α-glucosidase inhibitors, iminosugars and their synthetic derivatives (Nakagawa *et al*., 2010; Stocker *et al*., 2010; Zamoner *et al*., 2019), GLP-1 receptor agonists or DPP-4 inhibitors, sulfonylureas, insulin and thiazolidinediones (Bailey *et al*., 2016). Consequence of prolonged use of one (and their combination), antidiabetic or antihyperglycemic drugs is the occurrence of adverse side effects such as weight loss, nausea, diarrhea, vitamin B12 deficiency (Alhaji, 2022; Kibirige & Mwebaze, 2013), hypoglycemia, lipoatrophy, lipohypertrophy, vomiting and pancreatitis, which has led patients with diabetes to abandon treatment (Chaudhury *et al*., 2017). Therefore, the search for new molecules with minimal adverse effects for the treatment of diabetes is imperative.

In this context, medicinal plants have been a viable alternative to mitigate the adverse effects of drugs involved in the treatment of diabetes, as well as having molecules capable of exerting a hypoglycemic effect (Shabab *et al*., 2021). Worldwide, the use of *Aloe vera*, *Camellia sinensis*, *Capsicum annuum* and *Zingiber officinale* species (Bindu & Narendhirakannan, 2019) has become widespread as an alternative treatment for diabetes; in addition, species of the Asteraceae family such as *Artemisa ludoviciana*, *Cirsium mexicanum*, *Bidens pilosa*, *Brickellia cavanillesii*, *Iostephane heterophylla* and *Tithonia diversifolia* (Cilia-López *et al*., 2021) have been shown to have antihyperglycemic, antidiabetic and insulinotropic effects, results that support their ethnomedicinal use in some communities . These pharmacological effects have been attributed to secondary metabolites such as caffeic acid, gallic acid, quercetin, kaempferol, and rutin (Deka *et al*., 2022).

Among these medicinal species is found "Gordolobo" a bush of 60 to 150 cm in height, straight or slightly inclined stem, narrow and long leaves, its inflorescence has an arrangement of pods, with yellow (immature) and brown (pollinated) flowers. Its distribution and habitat are sub perennial tropical forests that cover central, northeastern, western, eastern, and southeastern parts of Mexico. Species identified in Mexican ethnomedicine are *Gnaphalium attenuatum* DC., *Gnaphalium oxyphyllum* DC., *Gnaphalium semiamplexicaule* DC. and *Gnaphalium viscosum* Kunth, which are an alternative treatment for diseases such as cough, asthma, throat infection, liver problems, as well to decrease high blood sugar levels and diabetes complications (UNAM, 2022). It has been proved and published that species such as *G. canescens* DC has gastroprotective effect against *Helicobacter pylori* at a mean inhibitory concentration (MIC) of 500 µg/mL for aqueous extract, and 62.5 µg/mL for methanolic extract of its aerial parts (Castillo-Juárez *et al*., 2009).Chemical composition studies described the presence of flavones such as 5,7-dihydroxy-3,8-dimethoxyflavone (gnaphaliin A) and 3,5-dihydroxy-7,8-dimethoxyflavone (gnapaliin B) which were isolated and identified from *G. liebmannii*, were each one shown relaxant properties on guinea pig tracheal smooth muscle; likewise, gnaphaliin A and gnaphaliin B are chemical-taxonomic markers in the identification of species of *Gnaphalium* genus according to Herbal Pharmacopoeia of the United Mexican States (Rodríguez-Ramos *et al*., 2009).

In South Korea, anti-inflammatory properties of *Gnaphalium affine* have been studied, and reports indicate that chloroform-soluble fraction of *G. affine* methanolic extract inhibits the expression of proteins (NF-κB and MAPKs) involved in acute inflammatory response signaling pathways in RAW264.7 cells stimulated with lipopolysaccharide (LPS) (Seong *et al*., 2016); likewise, flavonoids isolated from ethyl acetate-soluble fraction of *G. affine* methanolic extract dose-dependently inhibited the production and release of human neutrophil elastase (HNE), nitric oxide, induced nitric oxide synthase (iNOS), and proinflammatory cytokines that are in-volved in acute inflammatory response of respiratory diseases (Ryu *et al*., 2016).

Researchers in China have performed research on the folkloric use of the species *G. affine* highlight its inhibition of the enzyme xanthine oxidase, which is involved in diseases with an inflammatory and oxidative factor such as gout and arthritis, which has been attributed to flavonoids such as apigenin, luteolin, caffeoylquinic acid esters, kaempferol, and other polyphenolic compounds (Lin *et al*., 2014; Huang *et al*., 2015; Zhang *et al*., 2017; Zhang *et al*., 2018). *Gnaphalium hypoleucum* DC is another species that has been reported to have antimicrobial activity based on hindering the quorum sensing (QS) of antibiotic-resistant bacteria such as *Chromobacterium violaceum* (Li *et al*., 2022) and antiglycemic activity inhibiting the activity of the enzyme α-glucosidase in streptozotocin-induced diabetic Kunming mice (Sun *et al*., 2017).

Currently, research on medicinal plants has opened up multiple pharmacological applications of a plant species outside its traditional or ethnomedicinal use, so the objective of this study is to evaluate the use of "gordolobo" (*Gnaphalium sp*) on an *in vivo* model of alloxan-induced experimental diabetes, taking advantage of the polyphenolic compounds that may be present in the chloroformic extract of its aerial parts as well its acute toxicological evaluation in rats.

MATERIAL AND METHODS

Plant material

1 kg specimen was acquired in 2019 under the name "flor de gordolobo" and was identified as *Gnaphalim* sp (*Gna.* sp) lot number CD52078.2 from the company Central de Drogas, S. A. de C. V. located in La Perla Naucalpan, State of Mexico. Subsequently, aerial parts of the plant were subjected to particle reduction using a laboratory mill model PULVEX MINI-100 (Mexico City, Mexico) with a 1.5 h.p. single-phase rotor.

Preparation of the chloroformic extract of Gnaphalium sp

A maceration was performed using 300 g of ground plant material and 4.5 L of a mixture of chloroform ($CHCl_3$) (Fermont®, Monterrey, Nuevo León, Mexico) and distilled water (H_2O) (4:1) at room temperature for one week; the sample was subsequently filtered. When the phases were separated, organic phase was concentrated using a Buchi B-490 rotary evaporator (Flawil, Switzerland), and final extract obtained was dried with air. Chloroformic extract of *Gnaphalium sp* (CEG) obtained was stored at 8°C in dark for later use.

Preliminary TLC phytochemical analysis and HPLC

For thin layer chromatography (TLC) analysis a 1% solution was prepared using ground plant material (0.5 g of *Gnaphalium sp* in 50 mL of distilled water) that was allowed to dissolve for one week. Resulting filtrate was colorimetrically tested according to the methodology described by Lescas Nava *et al.* (2016), for qualitative identification of secondary metabolites. Silica gel 60 F254 plates (Merck Brand), pre-coated with aluminum, with a pore size of 0.2 mm, were used as stationary phase to be able to separate secondary metabolites of CGE based on their polarity, preparing the following elution systems, as mobile phase: for polar compounds: ethyl acetate, methanol, and water (100:13.5:10), and for non-polar compounds: toluene, ethyl acetate (97:3). Results were reported as abundant (+++), moderate (++), scarce (+) or null (-).

For high performance liquid chromatography (HPLC), an Agilent 1100 chromatograph (Santa Clara, California, USA) was used to identify the compounds in CEG. Fifty milligrams of CEG were weighed and dissolved in 3 mL of ethanol (Merck®, Darmstadt, Germany). A 20 µL aliquot was run on an Agilent Eclipse XDB C18 column (150 x 4.5 mm) (Santa Clara, California, USA) with a constant temperature of 25°C. A mixture of 95% 0.01 M phosphoric acid (H_3PO_4, solvent A) and 5% methanol (MetOH, solvent B) (Merck®, Darmstadt, Germany) was used as the mobile phase, and the concentration gradient of solvent B was adjusted to a constant flow rate of 1 mL/min. The assay was performed in triplicate (n=3).

Identification of CEG compounds by CG-MS

GC-MS analysis of CEG was carried out on a Aligent G2570A 6850 GC/MS system (Santa Clara, California, USA). Sample (1 g) was dissolved in 1 mL of chloroformic (Fermont®, Monterrey, Nuevo León, Mexico). Resuspended sample (1 µL) was injected through a capillary column Agilent 19091S-433E (Santa Clara, California, USA) having 30 m length, 0.25 mm inner diameter and 0.25 µm film thickness. Injector temperature was maintained at 250°C, 116 kPa pressure and 1.5 mL/min flow. Helium was used as a carrier gas, and total GC-MS program time was 20 min. Mass spectra were recorded, and compounds were identified using National Institute of Standards and Technology (NIST) mass spectral library.

Quantification of total polyphenolic compounds and flavonoids contents

A 1% solution of dried CEG was prepared for the quantification of polyphenolic compounds and flavonoids using the methodology described in Osorio-Esquivel *et al*. (2011), with modification, where Folin-Ciocalteu (Merck®, Darmstadt, Germany) phenolic reagent was used undiluted and for the quantification of flavonoids anhydrous aluminum chloride ($AlCl_3$) (Merck®, Darmstadt, Germany) was used changing the volume from 150 to 50 µL for the reaction. Polyphenolic compounds were quantified using gallic acid (Sigma-Aldrich®, St. Louis, Missouri, USA) [0-0.1 mg/mL] as a pure standard, and the results are reported as gallic acid equivalents (GAE)/g dried extract. For the quantification of flavonoids, catechin (Sigma-Aldrich®, St. Louis, Missouri, USA) [0-0.3 mg/mL] was used as a pure standard to generate a calibration curve, and results are reported as catechin equivalent (CE)/g dried extract. A Bio-mini Shimadzu UV-VIS spectrophotometer (Oregon, USA) was used for both tests.

Antioxidant capacity in vitro assays

A 1% solution of CEG was prepared for the assays. To measure reducing power capacity, a ferrous ion reducing power (FRAP) antioxidant assay was performed following the methodology described by Benzie & Strain (1996), using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich®, St. Louis, Missouri, USA) [0-1 mM] as an antioxidant standard for the calibration curve; results are reported as Trolox equivalent (TE)/g dried extract. Additionally, 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich®, St. Louis, Missouri, USA) (DPPH) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (Sigma-Aldrich®, St. Louis, Missouri, USA) (ABTS) were used to measure the percentage of radical inhibition (%). For DPPH assay (Brand-Williams *et al*., 1995), percentage of inhibition (%) was calculated with the following equation:

$$(\%)=\left[1-\left(\frac{\text{Abs sample}}{\text{Abs blank}}\right)\right]*100$$

Trolox [0-500 µM] was used as a reference standard for the calibration curve. For ABTS assay (Ozgen *et al*., 2006), percentage of inhibition (%) was calculated with the following equation:

$$(\%)=\left[1-\left(\frac{\text{Abs sample}}{0.7}\right)\right]*100$$

Trolox [0-400 µM] was used as a reference standard for the calibration curve. A Bio-mini Shimadzu UV-VIS spectrophotometer (Oregon, USA) was used for both in vitro assays.

Experimental animals

Male and female Wistar rats weighing between 180 and 210 g were used. Animals were housed in a room with a constant temperature of 25 ± 1°C and a 12-hour light/dark cycle. Animals were fed *ad libitum* with Laboratory Rodent Chow Diet 5001 (St. Louis, Missouri, USA) and purified water. The use of animals was approved by the Bioethics Committee of the National School of Biological Sciences of the National Polytechnic Institute under folio number ENCB/CEI/085/2020 CONBIÉTICA-09-CEI-002-20190327, guidelines described in NOM-062-ZOO-1999, modified in 2001, for the "Handling, Use, Storage and Disposal of Laboratory Animals" were also considered.

Acute oral toxicity

Organization for Economic Cooperation and Development (OECD, 2001) guideline 423 was used as a reference. A single initial dose of 2000 mg/kg CEG was administered intragastrically (*i.g*.) in a single time to 3 male and 3 female Wistar rats each, which were subsequently observed for posterior first at 4 h and then every 24 h for 14 days. Also, two experimental groups of each sex (n=3) were administered with the vehicle (tween 80: water, 1:9 ratio) by *i.g*. route in a single time, (10 mL/kg) as healthy control groups. After administration, the observation was carried out and the behavior of the animals was evaluated for 6 hours, then 24 and 48 hours, body weight was also recorded every third day. At the end of the study, animals were euthanized by cervical dislocation, and vital organs were removed for macroscopic analysis to search for lesions and discoloration in the tissues, as well to calculate relative weight ratio of each (%), using the following equation:

$$\% = \frac{(\text{Organ weight (g)})}{(\text{Animal weight (g)})} * 100$$

Alloxan-induced experimental diabetes

This model was established according to Elangovan *et al*. (2019), with alloxan (Cayman® Ann Arbor, Michigan, USA) dose modified to 170 mg/kg injected intraperitoneally (*i.p.*). Seventy-two hours after induction, blood´s glucose concentration was measured using One Touch Ultra® brand test strips and a glucometer (Malvern, Pennsylvania, USA) by taking blood samples from tail vein. Animals with values ≥300 mg/dL were considered diabetic and were used for the study (Gharib & Kouhsari, 2019).

Experimental design

A total of 36 Wistar rats (18 males and 18 females) were used for the 15-day model. Animals were randomly distributed by sex into the following three groups, with six animals per group (n=6): A) control healthy group (nondiabetic rats administered only purified water, 10 mL/kg); B) diabetic untreated group (diabetic rats administered only purified water, 10 mL/kg), C) diabetic CGE-treated [D + CEG] group (diabetic rats administered with CEG at 200 mg/kg). Water and extract were administered daily for 15 days *i.g*. using a stainless-steel cannula.

Glucose tolerance test and insulin curve

At the end of the 15-day model animals were fasted for 12 h after last administration of CEG. Blood's glucose concentrations were measured using glucose test strips and a One Touch Ultra® glucometer (Malvern, Pennsylvania, USA), whose value was considered basal concentration at zero minutes. Subsequently, a dose of 2 g/kg glucose (dextrose) (J. T. Baker®, Radnor, Pennsylvania, USA) was administered by *i.p*. route (Loza-Medrano *et al*., 2020), and blood's glucose concentration was measured at 5, 15, 30, 60, 90 and 120 minutes. At the same time, blood samples were taken by retroorbital sinus puncture, and centrifuged at 13 000 rpm for 15 minutes to obtain serum for quantification of insulin using rat/mouse insulin ELISA kit (Merck®, Darmstadt, Germany), as well triacylglycerides (TG) were measure from serum using a commercial kit (RANDOX brand), according to manufacturer's instructions.

Oxidative stress evaluation

After the blood samples have been collected, animals were euthanized by cervical dislocation, then pancreas and liver were extracted to perform a macroscopic evaluation and calculate relative weight ratio (%). For pancreas and liver, 0.5 g of each tissue was taken to determine two biomarkers of oxidative damage on biomolecules: lipid peroxidation rate through malondialdehyde (MDA) tissue concentration based on the methodology described by Buege & Aust (1978), using 2-thiobarbituric acid (TBA) (Cayman® Ann Arbor, Michigan, USA), as well as total protein oxidized (Pox) concentration based on the methodology described by Parvez & Raisuddin (2005), using 2,4-dinitrophenylhydrazine (Sigma-Aldrich®, St. Louis, Missouri, USA). For pancreas, RANDOX brand RANSOD and RANSEL kits (Crumlin, County Antrim, Northern Ireland, UK) were used for the quantification of antioxidant enzymes activity of superoxide dismutase (SOD), and glutathione peroxidase (GPx), respectively, both reported as IU enzyme/g following manufacturer's instructions. A Bio-mini Shimadzu spectrophotometer (Oregon, USA) was used to read all parameters.

Assessment of glucose metabolism homeostasis

The following equations from previous published work by Cersosimo *et al*. (2014), were used for calculations:

$$\text{HOMA-IR} = \frac{(20 * \text{fasting insulin concentration})}{(\text{fasting glucose concentration} - 3.5)}$$

$$\text{HOMA-}\beta = \frac{(\text{fasting insulin concentration} * \text{fasting glucose concentration})}{(22.5)}$$

$$\text{HOMA-S} = \frac{1}{\text{HOMA-IR}}$$

Statistical análisis

Data are presented as the mean (±), and its standard error of the mean (SEM). Significant differences between groups were determined by one-way ANOVA with Holm–Sidak post hoc test for parametric data and Kruskal–Wallis analysis of variance by ranks with Dunn post hoc test for nonparametric data using GraphPad Prism 8 statistical software. Results were considered statistically significant when $p<0.05$. Likewise, all results complied with normal distribution ($p>0.05$) by Shapiro-Wilk and Kolmogorov-Smirnov test.

RESULTS

Preliminary phytochemical screening by TLC, and HPLC analysis of Gnaphalium sp

Qualitative test results of preliminary phytochemical analysis (Table No. 1) showed that the most abundant secondary metabolites (+++) in CEG were sesquiterpene lactones, polyphenolic compounds, reducing sugars, triterpenes/steroids, and flavonoids.

Table No. 1
Phytochemical screening of *Gnaphalium sp* aerial parts

Identification tests	Metabolite to be identified	Results
Baljet	Sesquiterpene lactones	+++
Foam	Saponins	-
Rosenthaler	Triterpene saponins	-
Ferric chloride	Polyphenolic compounds	+++
Fehling	Reducing sugars	+++
Börntrager	Quinones and anthraquinones	+
Liebermann-Buchard	Triterpenes and steroids	+++
Dragendorff	Alkaloids	-
Hager	Alkaloids	-
Silicotungstic acid	Alkaloids	-
Shinoda	Flavonoids	+++
Liebermann-Buchard	Triterpenes and steroids	++
Baljet	Sesquiterpene lactones	++
Shinoda	Flavonoids	+
Börtrager	Quinones and anthraquinones	-

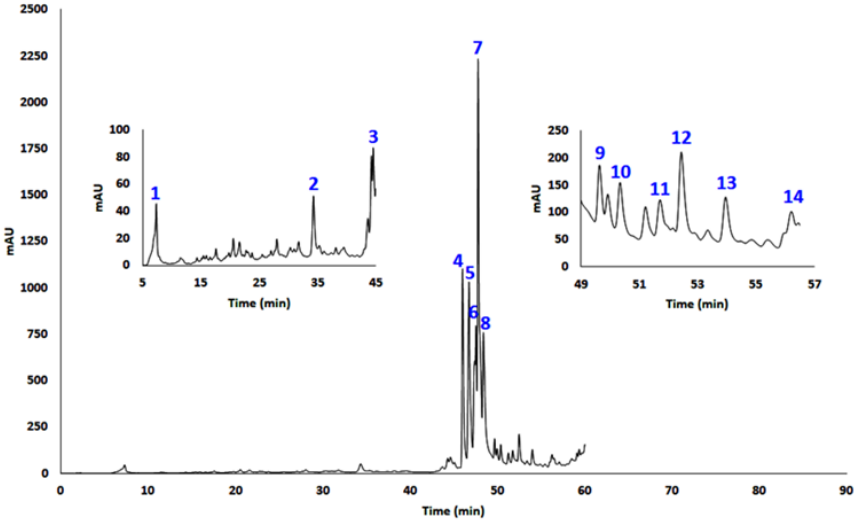
(+++) abundant, (++) medium, (+) low, (-) null

These phytochemical results are in agreement with those described by Zheng *et al*. (2013), where more than 125 isolated compounds were identifiedfrom several species of genus *Gnaphalium* by TLC means, and their biological activities were reported.

Meanwhile, Figure No. 1 showed HPLC chromatograph of CEG, which included 14

compounds (peaks with areas ≥ 1000 mAUC); of these peaks, peak 4 was identified as apigenin.

Figure No. 1
Reverse-phase chromatogram of the chloroformic extract of *Gnaphalium sp*



An enlargement of the chromatogram is shown for the retention times of peaks 1 to 3 and 9 to 14.

Additionally, peaks 4 to 8 are of great interest because of their abundance in CEG, peaks with the highest relative abundance (Ra) in descending order were: 7 (100%), 5 (54.4%), 4-apigenin (51.5%), 6 (41.2%) and 8 (28.2%), while all the other peaks showed values between 4.2 and 9.6%; all of these peaks share similar retention times (Rts) to apigenin (flavone); thus, they must have a similar chemical structure in the mobile phase (Table No. 2).

Table No. 2
Data obtained from reverse phase HPLC of CEG.

# Peak	Rt [1], (min)	Area (mAUC)	Mobile phase[2]	Identified compound	Ra [3], (%)
1	7.3	1672.0	95:5%	-	6.9
2	34.3	1284.3	60:40%	-	5.3
3	44.6	1261.6	50:50%	-	5.2
4	46.0	12461.0	30:70%	Apigenin	51.5
5	46.7	13148.6		-	54.4
6	47.5	9959.3		-	41.2
7	47.8	24185.3		-	100.0
8	48.4	6828.2		-	28.2
9	49.7	1034.6		-	4.3
10	50.3	1022.7		-	4.2
11	51.7	1037.6		-	4.3
12	52.5	2317.3		-	9.6
13	54.0	1185.0		-	4.9
14	56.2	1257.2	20:80%		5.2

[1]Retention time
[2]The ratio is phosphoric acid (H_3PO_4): methanol (MetOH)
[3]Relative abundance: Percentage ratio of the mAUC of the peaks shown in chromatogram with respect to the peak with the highest mAUC (area under curve) identified (peak 7).

Identification of chloroformic extract of Gnaphalium sp components by CG-MS

As a complement to the semi-quantitative phytochemical study of CEG, a gas chromatographic analysis coupled to mass spectrometry was performed. Results showed that CEG contains a total of 23 non-polar compounds as shown in the chromatogram (Figure No. 2).

From this chromatogram, 23 compounds were identified when compared each band to NIST database, concordances are shown in Table No. 3.

Table No. 3
Compounds identified using GC-MS from chloroformic extract of *Gnaphalium sp*

Peak	Rt (min)	Area %	Compound name	Ref NIST	CAS
1	3.5256	6.9367	Trichloromethane	8888	000067-66-3
2	4.1379	1.0981	n-Decanoic acid	38609	000334-48-5
3	5.1013	5.2735	Methyl 10-methyl-undecanoate	70183	1000336-21-1
4	5.3882	16.1388	Dodecanoic acid	59277	000143-07-7
5	5.5167	3.7277	Dodecanoic acid	59277	000143-07-7
6	5.7051	2.8872	Tridecanoic acid, methyl ester	81242	001731-88-0
7	6.0519	2.5646	2-Cyclohexen-1-one, 4-(3-hydroxy-1-butenyl)-3,5,5-trimethyl-, [R-[R*,R*-(E)]]	65543	052210-15-8
8	6.1376	3.1465	Nonadecane, 1-chloro-	138400	062016-76-6
9	6.296	3.0514	Tridecanoic acid, 12-methyl-, methyl ester	91870	005129-58-8
10	6.5529	6.4592	Tetradecanoic acid	81211	000544-63-8
11	6.6728	2.9469	Dodecanoic acid	59277	000143-07-7
12	6.964	3.4742	2-Pentadecanone, 6,10,14-trimethyl-	112033	000502-69-2
13	7.4136	1.5394	Methyl 10-methyl-dodecanoate	81240	1000336-24-9
14	7.4564	1.3138	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	118306	082304-66-3
15	8.5954	3.3572	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	132273	000112-63-0
16	9.7601	6.1405	Eicosane	122933	000112-95-8
17	13.8665	5.4344	Pentacosane	172981	000629-99-2
18	18.0158	-4.403	Methanone,[1,4-dimethyl-7-(1-methylethyl)-2-azulenyl]phenyl-	138552	039665-56-0
19	18.3027	7.2575	Eicosane	122933	000112-95-8
20	18.4268	2.2757	Palustric acid	138443	001945-53-5
21	18.5681	2.8634	(4-Ethylphenyl)-(2-methylbenzo[4,5]imidazo[1,2-a] pyrimidin-4-yl)amine	138356	1000322-09-4
22	18.7565	5.0478	Podocarpa,8,11,13-trien-3-one,14-isopropyl-13-methoxy-	147572	018326-16-4
23	20.8076	11.4685	Eicosane	122933	000112-95-8

Of these 23 compounds identified, Figure No. 3 shows the structures of those of greatest interest for this study (due to its possible beneficial effects during diabetes and its complications), highlighting some hydrocarbons, fatty acids, and their derivatives (esters), and others such as lactones and terpenoids.

Quantification of total polyphenolic, flavonoids, and antioxidant capacity of the chloroformic extract of Gnaphalium sp

Results showed that for total amount of polyphenolic compounds CEG contains 15.1 mg GAE/g dried extract, and from this, 46.3% are flavonoids, because there is 7.0 mg CE/g dried extract. For antioxidant capacity, FRAP assay results showed 489.3 mmol TE/g of dried extract, while for free radical inhibitory capacity, CEG had 100% inhibition over DPPH, while for ABTS assay, it was 89.60% at the tested concentrations (Table No. 4). In view of these data, CEG showed good antioxidant capacity *in vitro*.

Figure No. 2
CG-MS chromatogram for chloroformic extract of *Gnaphalium sp*

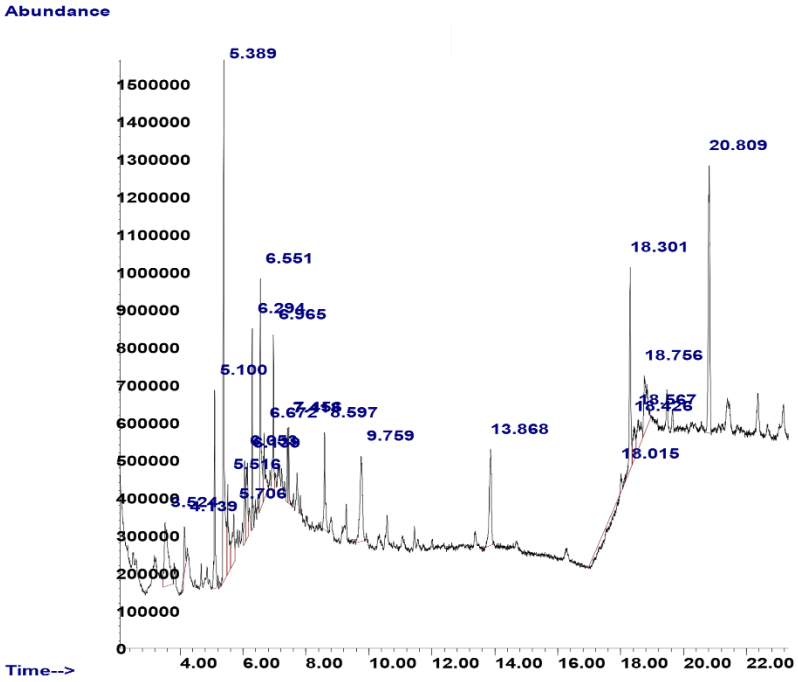


Figure No. 3
Structures of some compounds of interest identified in CEG by CG-MS

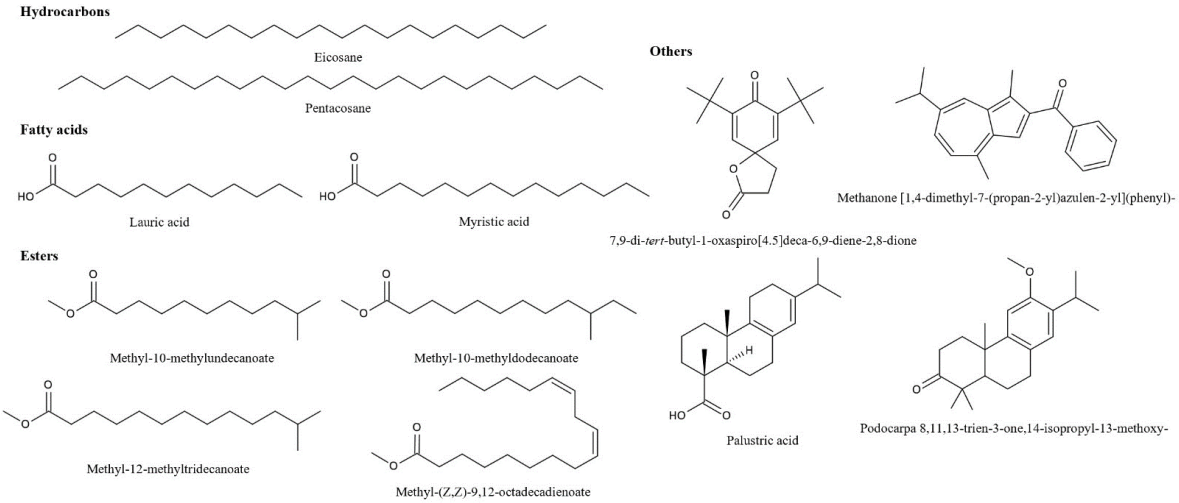


Table No. 3
Polyphenolic compounds, flavonoids, and antioxidant capacity of CEG

Phenolic	Flavonoids	FRAP	DPPH*	ABTS*
15.1 ± 0.1 mg GAE	7.0 ± 0.0 mg CE	489.3 ± 0.7 mmol TE	100 ± 0.0%	89.6 ± 0.4%

GAE: gallic acid equivalents; CE: catechin equivalents; TE: Trolox equivalents.
***Percentage of radical inhibition**.

Acute toxicity

Chloroformic extract of *Gnaphalium sp* at a single dose of 2000 mg/kg by *i.g*. route did not cause lethality in any animals at 6, 24 and 48 h, as well in any of the 14 days after administration. During this observation period, no signs of neurotoxicity, nor alterations in behavior were registered. Additionally, after euthanasia, and necropsy, no macroscopic alterations in aspect or relative weight were observed in major organs (data not showed). Considering all above-described data median lethal dose (LD_{50}) was estimated to be in the range of 2000 to 5000 mg/kg according to OECD, which indicates a category 5 compound in accordance with the criteria of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) in its latest update and publication ST/SG/AC.10/30/Rev.9 (UNECE and Europe, 2007).

Alloxan-induced diabetes model

Evaluation of hypoglycemic activity

In the groups with experimental diabetes, blood's glucose concentration increased almost in 38% for males, and 70% for females compared to values of control groups of healthy rats of each sex (137.8 ± 4.0 and 109.0 ± 9.8 mg/dL, respectively), which confirmed the development of alloxan-associated diabetes. Meanwhile, administration of CEG to diabetic animals did not decrease blood's glucose concentration in a significative manner neither in female nor male rats compared to diabetic un-treated diabetic groups of each sex; thus, CEG does not have hypoglycemic activity (Table No. 5). In the other hand, diabetic group of male rats, showed a significantly blood's TG concentration decrease of almost 74% (p=0.0130) compared to control healthy group (1.18 ± 0.24 mg/dL), while administration of CEG significantly increased TG's concentration in male rats in almost 270% (p=0.0130) compared to male un-treated diabetic rats (0.31 ± 0.15 mg/dL), and in female rats of almost 57% (p=0.0308) when compared to values showed by female un-treated diabetic rats (1.16 ± 0.07 mg/dL), where CEG-treated diabetic males restored serum values to those shown by healthy control (1.18 ± 0.24 mg/dL), however, the increase in females was greater than that of control group without diabetes or treatment (0.98 ± 0.13 mg/dL) (Table No. 5).

Table No. 4
Biochemical parameters and relative weight of adipose tissue in CEG-treated rats during experimental diabetes

		Control	Diabetic	D + CEG
Male	Glucose [mg/dL]	137.8 ± 4.0	190.0 ± 12.5†	239.5 ± 61.7
	Triacylglycerides [mg/dL]	1.18 ± 0.24	0.31 ± 0.15 †	1.17 ± 0.14 †
	Retroperitoneal WAT (%)	0.45 ± 0.06	0.39 ± 0.11	0.33 ± 0.14
Female	Glucose [mg/dL]	109.0 ± 9.8	187.0 ± 11.4†	498.5 ± 106.3
	Triacylglycerides [mg/dL]	0.98 ± 0.13	1.16 ± 0.07	1.82 ± 0.25 †
	Retroperitoneal WAT (%)	0.98 ± 0.11	1.68 ± 0.24	0.09 ± 0.06 *

Data are shown as the mean (±) SEM (n=6). One-way ANOVA with the Holm–Sidak multiple comparison test was applied for "Glucose", and "Triacylglycerides". Kruskal–Wallis ANOVA with Dunn's multiple comparison test was applied for "Retroperitoneal WAT". Significant differences are denoted as follows: †(p<0.05), *(p<0.001). WAT, white adipose tissue

For relative weight of retroperitoneal WAT results only in female diabetic group administered with CEG it decreased significantly in almost 95% (p=0.0008) compared to values of female diabetic un-treated group (1.68%), showing a value below of that showed by control healthy group (0.98%) (Table No. 5).

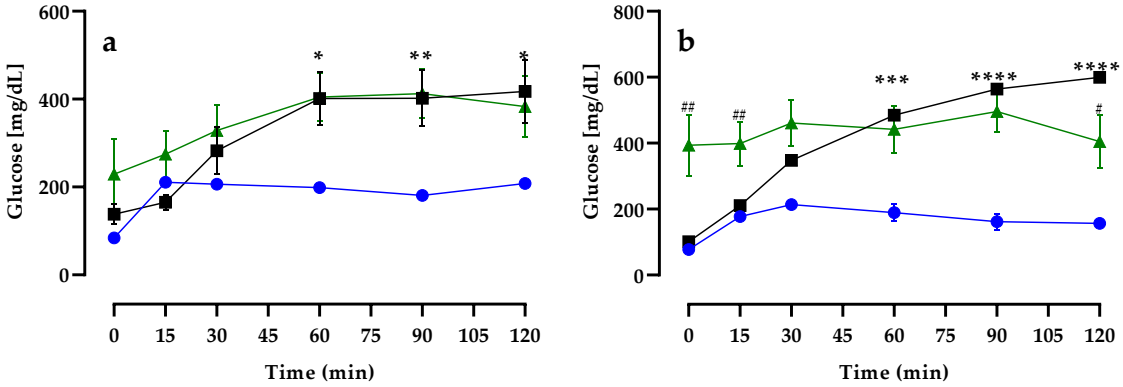
It has been reported that in biochemical blood's parameters associated with metabolism, such as blood's glucose, TG's, insulin, fatty acids, and cholesterol concentrations, both in clinical and preclinical studies, can be differences between individuals of different sexes because sex hormones are linked to metabolic regulation (Fourny *et al*., 2021).

In the context of hyperglycemia, another important indicator to consider is glucose tolerance. Healthy male rats of control group reached a maximum blood glucose concentration 15 minutes (211 ± 3.86 mg/dL) after a single intraperitoneal

administration of glucose, and this concentration decreased gradually over time in minutes 60 (198.83 ± 9.95 mg/dL), 90 (181 ± 3.80 mg/dL) and 120 (208.17 ± 10.25 mg/dL) (Figure No. 4a); meanwhile, for diabetic un-treated group, maximum blood's glucose concentration was reached at 60 minutes being a significance increase near to 102%, and remained constant from 90 to 120 minutes in almost 122 and 101%, respectively, compared to healthy male rats at same measure times. Likewise, diabetic group treated with CEG showed similar behavior to diabetic un-treated group, at 60, 90 and 120 minutes (404.50 ± 53.90, 412.67 ± 53.90 and 382.83 ± 70.14 mg/dL, respectively), being statically different only from healthy control group (Figure No. 4a).

For female rats, healthy control group reached maximal blood's glucose level at 30 minutes (214 ± 14.32 mg/dL), which gradually decreased in posterior measures at 60 (189.33 ± 26.63 mg/dL), 90 (162 ± 23.73 mg/dL) and 120 min (156.33 ± 17.41 mg/dL); meanwhile for un-treated diabetes group, maximal blood's glucose concentration was observed at 60 min a statically increase near to 155%, reaming for the next measure times of 90 (247%) and 120 (282%) minutes. Finally, for diabetic CEG-treated group, blood's glucose concentration was maintained during the 120 minutes of the test, however, at 120 minutes these values drop in a significance decrease of almost 33% with respect to that of untreated diabetic group at the same time (Figure No. 4b).

Figure No. 4
Intraperitoneal glucose tolerance test in alloxan-induced diabetic Wistar rats treated with CGE



a) Males and b) Females. Data are shown as the mean (±) SEM (n=6). One-way ANOVA with Holm–Sidak multiple comparison test. Significant differences are shown as follows: control vs. diabetic: *(p<0.05), **(p<0.01), *(p<0.001), ****(p<0.0001); and diabetic vs. diabetic + CEG: # (p<0.05), ## (p<0.01).**
● Control, ■ Diabetic, ▲ Diabetic + CEG; (n=6).

As a complementary evaluation to glucose overload in the tolerance curve, blood's insulin concentration was monitored, as shown in Figure 5. Results showed that for healthy control groups in both sexes, maximum concentration of insulin was reached at 15 minutes for male rats (5.82 ± 0.70 ng/mL) (Figure No. 5a) and same behavior was observed in healthy female rats (2.69 ± 0.38 ng/mL), however this increase only matin at 30 minutes for healthy female rats (3.98 ± 0.27 ng/mL) (Figure No. 5b), there was a decrease over time related as previously described with a gradual decrease in blood's glucose in healthy rats of both sexes, in contrast, diabetic un-treated groups, did not show an increase of insulin despite the fact that blood glucose levels were elevated at all measurement times. Likewise, diabetic CEG-treated group showed the same behavior as diabetic rats only administered with the vehicle, showing in both sexes a decrease in insulin levels of approximately ≈25% at 15 minutes compared to the values shown by the healthy control groups in both sexes at the same time of measurement, despite the increase in blood glucose.

Assessment of antioxidant activity in experimental diabetes model

In this study, alloxan was used to induce diabetes by damaging pancreatic beta cells via a molecular mechanism linked to oxidative stress, thereby altering insulin synthesis and its secretion (Ighodaro & Akinloye, 2018); parameters such as oxidative stress biomarkers levels and antioxidant enzyme activity are shown in Table No. 6. These parameters could be

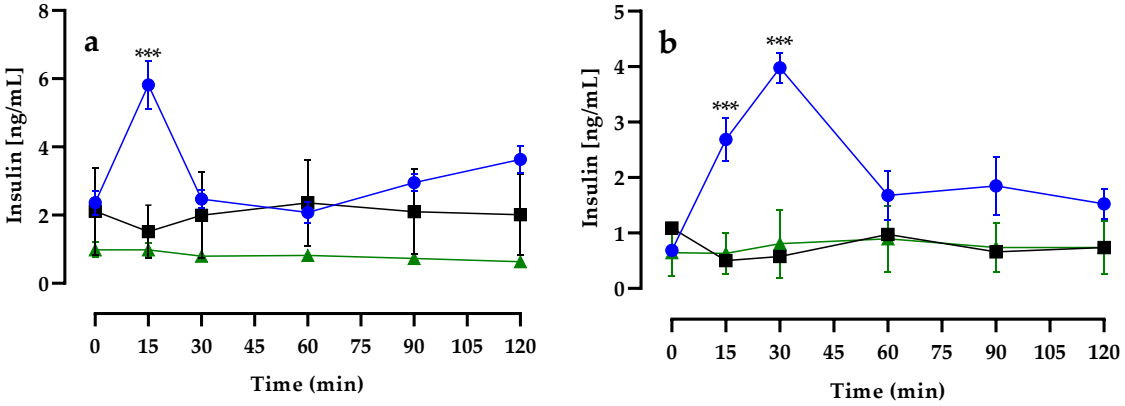
used to evaluate the oxidative damage that alloxan generates to establish experimental diabetes models in both sexes of rats.

Table No. 5
Pancreatic tissue oxidative damage biomarkers during alloxan-induced experimental diabetes in rats treated with CGE

		Control	Diabetic	Diabetic + CEG
Male	Relative weight (%)	0.5 ± 0.01	0.5 ± 0.05	0.5 ± 0.03
	MDA [nmol/g]	4.0 ± 0.6	20.1 ± 5.8*	89.2 ± 25.5
	POx [nmol/g]	0.2 ± 0.03	0.5 ± 0.2	0.2 ± 0.07
	SOD [U/mL]	772 ± 7	1547 ± 259*	1783 ± 280
	GPx [U/mL]	978.2 ± 21.4	722.7 ± 181*	689.7 ± 200
Female	Relative weight (%)	0.3 ± 0.05	0.5 ± 0.03	0.5 ± 0.04
	MDA [nmol/g]	1.1 ± 0.8	27.4 ± 6.6 *	1.8 ± 0.6 *
	POx [nmol/g]	0.8 ± 0.01	0.3 ± 0.05	0.2 ± 0.09
	SOD [U/mL]	1026 ± 123	2079 ± 68 #	1461 ± 261 †
	GPx [U/mL]	849.9 ± 143	350.1 ± 9.8*	486.0 ± 257*

Data are shown as the mean (±) SEM (n=6). Kruskal–Wallis ANOVA with Dunn's multiple comparison test was applied for "relative weight". One-way ANOVA with the Holm–Sidak multiple comparison test was applied for "MDA", "POx", "SOD" and "GPx". Significant differences are denoted as follows: †(p<0.05), #(p<0.01), *(p<0.001). MDA, malondialdehyde; POx, oxidized proteins; SOD, superoxide dismutase; GPx, glutathione peroxidase

Figure No. 5
Insulin blood levels in glucose tolerance test in alloxan-induced diabetic Wistar rats treated with CGE



a) Males and b) Females. Data are shown as the mean (±) SEM (n=6). One-way ANOVA with Holm–Sidak multiple com-parison test. Significant differences are shown as follows: control vs. diabetic: * (p<0.05), ** (p<0.01), * (p<0.001), **** (p<0.0001); and diabetic vs. diabetic + CEG: # (p<0.05), ## (p<0.01).**
● Control, ■ Diabetic, ▲ Diabetic + CEG; (n=6).

Relative weight of pancreas is an indirect parameter of inflammation, Table No. 6 shows that for the three groups of male and female rats, pancreas weight was constant and there were no significant differences. Oxidative damage derived from alloxan injection led to a significative increase only for malondialdehyde (MDA) biomarker in pancreatic tissue of diabetic non-treated groups for male and female rats compared to healthy controls. CGE-treatment decreased the MDA concentration only in pancreas of diabetic female rats (p=0.0007) (Table No. 6). Likewise, alloxan induction significatively increased SOD activity in pancreatic tissue of diabetic male and female rats (in almost ≈103% for

both sexes), and decreased GPx activity (near to 26% for males, and 58% for females) compared to healthy controls in both cases; finally, CEG administration significantly decreased SOD activity (29%) and increased GPx activity (39%), but only in diabetic female rats, compared to diabetic un-treated control group values (Table No. 6).

Assessment of glucose metabolism homeostasis

Since diabetes is a systemic disease, liver damage is one of its associated physiological complications. Table No. 7 showed that relative weight of diabetic untreated and CEG administered groups did not show any increase compared to values of healthy control groups in both sexes. Regarding oxidative stress biomarkers (Table No. 7), only in female rats a statically increase in hepatic MDA concentration (almost 50%) and POx levels (near to 70%) was observed for diabetic nontreated group, and this difference was significant compared to healthy female rats (7.7 ± 1.0, and 0.2 ± 0.02 nmol/g, respectively). Similarly, CEG 15-day treatment significantly decreased MDA levels only in female rats (38%), as well as the POx concentration (43%), in the liver during experimental diabetes (Table No. 7).

Table No. 6
Liver tissue oxidative damage biomarkers during alloxan-induced experimental diabetes in rats treated with CGE

		Control	Diabetic	Diabetic + CEG
Male	Relative weight (%)	3.4 ± 0.01	3.7 ± 0.1	3.8 ± 0.2
	MDA [nmol/g]	6.5 ± 0.4	8.0 ± 2.0	10.4 ± 3.9
	POx [nmol/g]	0.2 ± 0.02	0.1 ± 0.03	0.1 ± 0.03
Female	Relative weight (%)	3.3 ± 0.1	3.5 ± 0.01	4.9 ± 0.2
	MDA [nmol/g]	7.7 ± 1.0	14.7 ± 0.5 #	9.0 ± 2.0 †
	POx [nmol/g]	0.2 ± 0.02	0.14 ± 0.02#	0.2 ± 0.03†

Data are shown as the mean (±) SEM (n=6). Kruskal–Wallis ANOVA with Dunn's multiple comparison test was applied for "relative weight". One-way ANOVA with the Holm–Sidak multiple comparison test was applied for "MDA" and "POx". Significant differences are denoted as follows: †(p<0.05), # (p<0.01). MDA, malondialdehyde; POx, oxidized proteins; SOD, superoxide dismutase; GPx, glutathione peroxidase

Finally, Table No. 8 shows HOMA index describing beta cell function (HOMA-B), insulin resistance (HOMA-IR) and insulin sensitivity (HOMA-S).

Table No. 8
Parameters associated with glucose metabolism homeostasis.

		Control	Diabetic	Diabetic + CEG
Male	HOMA-β	1123.0 ± 76.3	398.0 ± 193.4	203.5 ± 66.7
	HOMA-IR	14.6 ± 2.5	27.4 ± 20.5	11.9 ± 2.3
	HOMA-S	0.11 ± 0.03	0.14 ± 0.03	0.11 ± 0.02
Female	HOMA-β	1498.0 ± 573.9	297.8 ± 0.6	117.6 ± 148.6
	HOMA-IR	4.0 ± 0.9	8.0 ± 0.02	6.0 ± 2.5
	HOMA-S	0.29 ± 0.04	0.13 ± 0.0004	0.37 ± 0.12

Data are shown as the mean (±) SEM (n=6). One-way ANOVA with Holm–Sidak multiple com-parison test

In both diabetic untreated groups, HOMA-B index was not significantly different compared to that of healthy control groups, as well as for diabetic groups administered CEG for 15 days; however, although not significant, a downward trend was observed in diabetic groups with and without

treatment in both sexes, which suggests that alloxan's damage to pancreas β cells was irreversible. Additionally, HOMA-IR index in diabetic nontreated groups showed a nonsignificant increase in insulin resistance compared to healthy control groups for both sexes, and diabetic groups that received CEG treatment also showed a nonsignificant decrease in HOMA-IR index in both sexes. Finally, HOMA-S index depends on biological sex of rats (Table No. 8). Male rats with untreated diabetes had a nonsignificant increase in HOMA-S index compared to healthy control group. HOMA-S index decreased in diabetic group that was given CEG, but difference was not significant. In female diabetic untreated rats, HOMA-S index was lower than those values of normal rat control group, but not significantly, and with CEG administration during experimental diabetes HOMA-S index increased visually, even surpassing that of control group of healthy female rats (Table No. 8).

DISCUSSION

Ethnopharmacological and ethomedicinal research on "gordolobo" species that have been used as herbal remedies for alternative treatment of chronic-degenerative diseases such as diabetes in Mexico, as well as scientific verification of its biological activities like antidiabetic or hypoglycemic effects, that clarify its true medicinal potential are still today scarce; however, in general, it has been reported that in Mexico, there are several species of the genus *Gnaphalium* (family Asteraceae) that are distributed in different parts of the Mexican territory (*G. sp*. [Veracruz], *G. canescens* DC. [Tamaulipas], G. *purpurascens* DC. [Oaxaca] and *G. viscosum* Kunth [Chihuahua]); the primary use of these plants by the Mexican population of each region is to treat or alleviate respiratory diseases (Carr *et al*., 1999).

Other biological activities of different types of extracts from species of this genus are currently being investigated; for example, Rojas *et al*. (2001), evaluated the antimicrobial activity of the hexanic, methanolic and chloroformic extracts of *G. oxyphyllum* DC. and *G. americanum* Mill (Morelos) on bacterial strains of *Staphylococcus aureus*, *Streptococcus pneumoniae* and *pyogenes*, *Enterococcus faecalis*, *Escherichia coli* and *Candida albicans* in 2001. Furthermore, Villagómez-Ibarra *et al.* (2001), reported the presence of luteolin and 3-methoxyquercetin in the methanolic extract of flowers of *G. oxyphyllum*, as well 5-hydroxy-3,7-dimethoxyflavone in the hexanic extract of flowers *G. viscosum*; finally, β-sitosterol and stigmasterol were identified in the hexanic extract of the leaves and flowers of *G. oxyphyllum, G. liebmannii* and *G. viscosum* (Campos-Bedolla *et al*., 2005), being all of these secondary metabolites compounds that have great antioxidant capacity, responsible for various biological activities and therapeutic effects attributed to medicinal plants, and many of these families of bioactive metabolites were identified by TLC in the CEG in the present study.

Regarding the total content of polyphenolic compounds, Zeng *et al.* (2013), reported that methanolic extract of *G. affine* contained 154.36 mg GAE/g dried extract and 56.54 mg rutin equivalents/g dried extract, a ratio that agrees with our results that are shown in Table No. 3. Polyphenolic compounds refer to secondary metabolites that possess aromatic ring structures (C_6H_6) and hydroxy groups (-OH) (Handique & Baruah, 2002), and upon reaction with Folin's reagent (W^{6+}/Mo^{6+}), one of the enol type substituents becomes a keto group, favoring the delocalization of the double bonds of the aromatic ring, which allows for the transfer of electrons to form a coordination bond and the acquisition of a blue coloration (Ford *et al*., 2019). Meanwhile, flavonoids are a subtype of phenolic compounds whose main characteristic is: 1) a ring structure made up of a benzene (A-ring) fused with a tetrahydropyran (C-ring), which in turn forms a bond with a benzene (B-ring) at C2, and 2) substitution by hydroxy (-OH), oxo (=O), methoxy ($-OCH_3$) and carbohydrate (monosaccharides and oligosaccharides) groups on the A and B rings; these changes give rise to a wide variety of flavonoids (Fraga & Oteiza, 2011), and as a result, the amount of flavonoids is specific and small in comparison with the total amount of phenolic compounds that an extract may contain as was found in the present study.

Polyphenolic compounds and flavonoids have excellent antioxidant activity in a wide variety of biological applications (Panche *et al*., 2016), so determining the antioxidant capacity of extracts that contain them is essential. Zeng *et al*. (2013), reported that methanolic extract of *G. affine*, inhibited DPPH radicals by 85.43% and ABTS radicals by 86.90%, and after fractionation in a chromatographic column Sephadex LH-20 the 80% methanol subfraction inhibited DPPH radicals by 82.32% and ABTS radicals by 95.27%. These previously published results are comparable with those of the present study as CEG inhibited DPPH radical formation by 100%,

and ATBS radical formation by 89.6%; our results also showed that CEG contains 489.3 mmol Trolox equivalents/g dried extract based on FRAP assay results, this last result exhibit CEG great antioxidant capacity as a reducing agent over transition metals such as iron (Fe^{3+}/Fe^{2+}) which, if they are in the organisms in their reduced form, will not be able to exacerbate or favor the generation of reactive oxygen species (ROS), such as the hydroxyl radical (•OH) (Munteanu & Apetrei, 2021); all these results support CEG potential to regulate electron donors in stable radicals directly in its chemical structure such as DPPH (Kedare & Singh, 2011), and radicals formed by oxidizing agents ($ABTS^{+}$) (Shi *et al*., 2022). Thus, antioxidant capacity of CEG has been mostly attributed to secondary metabolites such as flavonoids and other compounds such as sesquiterpene lactones, triterpenes, and steroids, which were found by TLC (Table No. 1).

Flavonoids such as apigenin, luteolin, kaempferol, quercetin, rutin, gnaphalin B and gnaphalin A are some flavonoids that have been isolated and identified in species such as *G. affine*, *G. luteumalbum*, *G. sylvaticum*, *G. uniflorum*, *G. stramineum* and *G. liebmannii* (Rastrelli *et al*., 1998; Aquino *et al*., 2002; Konopleva *et al*., 2006; Xi *et al*., 2012; Al-Snafi, 2019); meanwhile, triterpenes such as α and β-amyrin, betulinic acid, squalene and phytosterols such as β-sitosterol and stigmasterol have been found in species such as *G. affine, G. gaudichaudianum* and *G. inornatum* (Meragelman *et al*., 2003; Osti-Castillo *et al*., 2010; Xi *et al*., 2011). Ontiveros-Rodríguez *et al.* (2022), with a methodology for the preparation of CEG similar to that was used in the present study, reported the presence of 4 flavones (gnaphalin A, gnaphalin B, araneol and 3,5,7-tri-O-methylgalangin) that were isolated, purified and identified by nuclear magnetic resonance (NMR); diterpenes sclareol and kaur-16-en-18-oic acid were identified also by NMR-HSQC of several samples of the "gordolobo" plant that were acquired at different points of sale in Mexico City.

As stated above, HPLC and GC-MS results showing CEG phytochemical profile in Figure 1 and Figure No. 2**,** respectively, indicate the presence of flavonoids for peaks 5 to 8, and for peak 4 apigenin was identified. Regarding other metabolites, some of them may be of high polarity found at low concentrations (peaks 1, 2 or 3) such as derivatives of caffeoylquinic acids (chlorogenic acid and cynarin) (Shikov *et al*., 2010). Remaining compounds (peaks 9 to 14) with lower polarity may be examples like germacrene D (Bohlmann & Ziesche, 1980), sclareol (Garcia *et al*., 1982), kauranol (Torrenegra and Angarita, 1977) or sylviside (Konopleva *et al*., 2006). Volatile compounds detected by CG-MS (Figure No. 2), eicosane and pentacosane are reported as major metabolites in essential oils of medicinal plants such as *Flos Chrysanthemi indici* (Asteraceae) (Jing *et al*., 2019), *Ginkgo biloba* L. (Ginkgoaceae) (Czigle *et al*., 2019) and *Calendula officinalis* L. (Asteraceae) (Ak *et al*., 2021). Low polarity of these hydrocarbons makes their extraction with chloroform feasible. Studies have reported possible leishmanicidal (Delgado-Altamirano *et al*., 2019) and antinociceptive (Cruz-Salomon *et al*., 2022) activity of eicosane, as well as antibacterial (Arora & Meena, 2017) for pentacosane. Fatty acids and their derivatives (esters) are similarly abundant metabolites in plant species without flowers (*Arabidopsis thaliana* (Brassicaceae), *Petroselium crispum* (Apiaceae), and *Solanum tuberosum* (Solanaceae) (He *et al*., 2020), and with flowers such as *Hibiscus sabdariffa* L. (Malvaceae), *Matricaria recutita* L. (Asteraceae) and *Mentha aquatica* L. (Lamiaceae) (Fernandes *et al*., 2018), to name a few. Lauric acid is attributed with antibacterial, antifungal, antiviral, anticancer, and cholesterol, body weight and cardiovascular disease lowering activities (Sandhya *et al*., 2016). Myristic acid is an essential component in the cell membranes of (human) cells having the function of a lipid-anchored protein; likewise, it has been reported to possess antifungal, antiviral, anticancer, antiparasitic and immunomodulatory properties (Javid *et al*., 2020). Metabolite 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione has been identified in the n-hexane fraction of the ethyl acetate fraction of methanolic extract of whole plant of *Euphorbia pulcherrima* (Euphorbiaceae), giving it the classification of flavonoid (Sharif *et al*., 2015); in the stem bark of *Manilkara hexandra* (Roxb.) (Sapotaceae), (Monisha & Vimala, 2018); and in the ethanolic extract of the seaweed *Chara baltica* (Characeae) (Tatipamula *et al*., 2019). Methanone,[1,4-dimethyl-7-(1-methylethyl)-2-azulenyl]phenyl- has been identified in methanolic extract of *Vernonia cinerea* (syn. *Cyanthillium cinereum*) (Asteraceae) showing an antibacterial effect against *Xanthomonas oryzae* pv. *Oryzae (Xoo)* in an *in silico* study (Joshi *et al*., 2021) and in ethanolic extract of *Allium sativum* L. (Amaryllidaceae) bulbs, where it is proposed as a potential drug against lung cancer (Padmini *et al*., 2020). Of the compounds identified in CEG, presence

of palustric acid (diterpenoid) in CEG is interesting as its abundance is common in trees of the Pinaceae family (*Abies balsamea*, *Pinus strobus*, *Picea glauca*, *Pinus banksiana*, *Picea mariana*) (Waye *et al*., 2014); however, its presence has been reported in extracts obtained by maceration of several plant species (Leguminosae [Fabaceae], Compositae, [Asteraceae], Myrtacea and Rubiaceae (Ratter *et al*., 1997)) from the Cerrado region (Brazil), evaluating its cytotoxicity in the Mac Coy mouse fibroblast cell line (Soares *et al*., 2011), as well as its evaluation as an immunomodulator for hydrogen peroxide (H_2O_2) generation in mouse peritoneal macrophages (Moreira *et al*., 2001). Finally, podocarpa,8,11,13-trien-3-one,14-isopropyl-13-methoxy- has been identified in the leaves of the ethanolic extract of *Scabiosa columbaria* L. (Caprifoliaceae) with the classification of steroid (Sagbo *et al*., 2020), it was also found in alcoholic extract of some herbal formulation consisting of stem barks of *Azadirachta indica* A. Juss (Meliaceae). and *Saraca asoca* Roxb. species (Fabaceae) where it is documented to possess properties for alleviating symptoms of common respiratory diseases and for the treatment of spontaneous abortion and preventing premature birth (Gupta *et al*., 2019).

Being that in Mexico *Gnaphalium sp* it is also part of various formulations and over-the-counter herbal products, the toxicological evaluation of the CEG was carried out because there are currently few toxicity, lethality and safety studies of the species, one of them Déciga-Campos *et al.* (2007), reported that methanolic and methylene chloride (MetOH:CH_2Cl_2; 1:1) extracts had an LD_{50} of 2852 mg/kg in male ICR mice by single intragastric administration, results related to those of the present research where CEG was not shown to be neurotoxic (absence of alkaloids), or lethal at a single dose of 2000 mg/kg during acute oral toxicity evaluation in Wistar rats based on single i.g. administration; median lethal concentration (LC_{50}) was also evaluated with a value of >1000 µg/L for *Artemia salina*; simultaneously, authors showed that *Gnaphalium sp* extract induced mutations in *Salmonella typhimurium* at a concentration of 1000 µg/plate, increasing the number of revertant colonies (mutagenic index, MI) in the TA 98 strain without (-) and with (+) S9 fraction (MI 101.0 and 132.6, respectively) with possible mutagenic potential, however, this index was below that of the positive control where the MI in TA 98 -S9 was 398 following induction with picrolonic acid (PA), and MI in TA 98 +S9 was 5416.9 following induction with 2-aminoanthracene (2-AA). Another study of cytotoxicity that was carried out by Campos-Bedolla *et al.* (2005), reported an LC_{50} > 400 µg/mL in mouse spleen cell culture and that 6400 µg/mL of methanolic extract of *Gnaphalium conoideum* where maximum tested concentration caused 70% mortality in cultured cells.

Scientific research to support the ethnomedicinal use of "gordolobo" (*Gnaphalium sp*) for diabetes treatment there not have been report yet, only for the treatment of respiratory diseases; however, a vast amount of previous research has shown that polyphenolic compounds are responsible for the biological activity of medicinal plants, including the ability to reduce blood's glucose levels in people with diabetes (Andrade-Cetto & Heinrich, 2005). As shown in this work, CEG contains a large amount of polyphenolic compounds that could help reduce hyperglycemia that occurs with diabetes. Despite this hypothesis, results showed that CEG did not decrease blood's glucose concentrations in diabetic CGE-treated rats. Elevation of blood's glucose in diabetic rats injected with alloxan is due to low concentration of insulin in blood caused by its toxicological mechanism of damage over pancreatic β cells (Rohilla & Ali, 2012) through oxidative stress, and this generated an adverse impact as well in insulin-sensitive or insulin-dependent organs (liver, skeletal muscle, and adipose tissue) (Honka *et al*., 2018).

Blood's glucose increase observed in animals administered CEG, may be due to metabolic stress, which can be defined as "...disequilibrium in homeostasis of a living organism as a consequence of an abnormal utilization of nutrients...but more frequently of pathological or physiological conditions, which can alter the intake, assimilation and/or correct utilization of nutrients" (Lacetera, 2016); CEG-treated diabetic rats compensate for the lack of glucose in their tissues by activating metabolic processes such as lipolysis, which hydrolyzes TG stored in adipose tissue to free fatty acids and glycerol (Bolsoni-Lopes & Alonso-Vale, 2015), which is used as a substrate for gluconeogenesis activation, leading to the synthesis of glucose in the liver and its release into the bloodstream (Rotondo *et al*., 2017). This hypothesis agrees with the fact that in the results of this work a decrease in TAG was observed in diabetic rats.

A low concentration or lack of blood's insulin (Figure No. 2 and Figure No. 3) could

promote lipolysis, so hormone-sensitive lipase (HSL) is active and favors TAG hydrolysis (Cignarelli *et al*., 2019), which leads to an increase in free fatty acids and glycerol that are then stored in the liver. Transcription factors that regulate gluconeogenesis are inhibited; for example, AKT (proteinkinase B)-mediated phosphorylation, CREB (cAMP response element-binding) transcription and PGC-1 transcriptional activity (Hatting *et al*., 2018).

Relationship between the proposed mechanism underlying the increase in blood's glucose concentration and CEG administration, is that secondary metabolites contained in CEG (polyphenolic compounds/flavonoids and fatty acids) favor increased lipolysis. Iwashita *et al.* (2001), reported 100 µM quercetin increased glycerol concentration in 3T3-L1 adipocyte culture medium, and Wang *et al.* (2018), reported that epigallocatechin gallate and chlorogenic acid increased lipolysis in adipose tissue isolated from male Sprague–Dawley rats. Kim *et al.* (2020), concluded that isoquercetin isolated from *Acer okamotoanum* (Sapindaceae) decreased the expression of genes associated with the transcription factors CEBPα, CEBPβ and PPARγ and lipogenesis-related proteins (FAS, ap2 and GLUT4) meanwhile, expression of lipolytic genes associated with ATGL and HSL was promoted by increased AMPK signaling. Additionally, it has been reported that apigenin (found by HPLC in CEG) favors the increase of SIRT1 in insulin-deficient cells and inhibits factors associated with lipogenesis such as STAT3, CD38, CD36 and PPARγ favoring lipolysis of TG's (Alam *et al*., 2021). Similarly, Bumke-Vogt *et al*. (2014), report that intracellular translocation of the forkhead box transcription factor O1 (FOXO1) increases in a dose-dependent manner with apigenin concentration in HepG2 (human hepatoma) cells. FOXO1 participates in hepatic glucose production when insulin concentration is low, with induction in the transcription of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pc) enzymes involved in the processes of gluconeogenesis and glycogenolysis. Additionally, it has been reported that lauric acid favors the mobilization of lipids stored in adipose tissue for its oxidation in liver, proposing the mechanism through the AMP-activated protein kinase-acetyl CoA carboxylase pathway, and promotion of gluconeogenesis in liver (Guo *et al*., 2023). Another studies demonstrated the effect of myristic acid on diabetes including Takato *et al.* (2017), where authors reported that a chronic intake of this MCFA favors, insulin resistance and body weight loss in NSY mice with type 2 diabetes, proposing the mechanism on the regulation of the activity of diacylglycerol kinase δ (DGKδ), an enzyme found in the B cells of the pancreas and where its relevance in their cellular proliferation has been observed (Sato *et al*., 2021).

However, to demonstrate these assumptions, further studies are required where the objective is to elucidate molecular mechanisms and interconnections between dyslipidemia and hyperglycemia in a metabolic syndrome model. Since elevated blood's glucose concentration is a trigger for metabolic stress, oxidative stress is common, and relevant manifestations are associated with diabetes and its adverse physiological effects.

Oxidative stress in diabetic rats was manifested as an increase in MDA concentration in pancreatic tissue, because of peroxidation of unsaturated lipids, and membrane phospholipids (Velazquez *et al*., 2011). Unsaturated lipids are part of cell membrane of pancreatic β cells, and peroxidation results from accelerated superoxide anion ($O_2^{\bullet-}$) formation based on the oxidation-reduction cycle of alloxan and its reactive metabolites alloxan radical ($AH^{\bullet}$) and dialuric acid (A). Loss of membrane integrity of β cells may be linked to low or no insulin secretion due to poor stability or reduced support from membrane proteins such as type 2 glucose transporters (GLUT2), ATP-sensitive potassium channels (KATP) and voltage-dependent calcium channels (VDCC) (Castiello *et al*., 2016). $O_2^{\bullet-}$ are the most reactive ROS, as well $HO^{\bullet}$, and although they play a role in immune defense, their electronic instability causes them to react with other biomolecules; moreover, they are precursors of other ROS, such as $HO^{\bullet}$, peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2) (Andrés *et al*., 2023). The increase in the activity of SOD enzyme is relevant, as this is the only antioxidant endogenous defense enzyme that can decrease tissular and cellular $O_2^{\bullet-}$ concentration, thereby preventing or decreasing the amount of oxidative damage caused by ROS (Younus, 2018).

Proteins are biomolecules that are susceptible to oxidative damage; therefore, an elevated POx concentration is a useful biomarker in chronic-degenerative diseases with an oxidative physiopathology development such as diabetes (Telci *et al*., 2000). Damage to proteins can be a result of multiple targets as follows: oxidation of residues with

thiol and aromatic groups, glycoxidation, lipoperoxidation, carbonylation and nitration/nitrosylation (Kehm *et al*., 2021). Therefore, quantification of POx, whose residues were oxidized to form carbonyl groups (Tamarit *et al*., 2012), was used to determine the impact of alloxan-induced diabetes. Reduced glutathione (GSH) is a very important tripeptide that is a nonenzymatic endogenous antioxidant and an important coenzyme for the antioxidant activity of GPx enzyme, which converts H_2O_2 to water (H_2O) (Pizzorno, 2014) to decrease the oxidative damage to tissues, such that exerted over β cells of the pancreas, by ROS; thus, low GPx activity is directly related to low GSH concentration. Studies such as that by Goyal *et al.* (2011), showed that GPx activity is low in obese diabetic patients; also, Altuhafi *et al.* (2021), reported that in people with diabetic nephropathy, GPx activity is lower than that in people with diabetes alone, but similarly, GPx activity in people with diabetes is lower than that in healthy people. Administration of CEG to diabetic rats led to a decrease in MDA concentration and POx and SOD activity but an increase in GPx activity; the proposed mechanism underlying these changes is ROS directly inhibition, which in general prevents the peroxidation of unsaturated lipids. For enzymes results, inhibition of $O_2^{\bullet-}$ favors the low activity of SOD, and with respect to GPx activity, this effect is augmented by the increased GSH concentration, as well by the decrease of its oxidized form (GSSG) (Sabu *et al*., 2002; Hegde *et al*., 2005; Yuldasheva *et al*., 2016; Albasher *et al*., 2020).

Oxidative damage to β cells of the pancreas reduces or stops insulin secretion, and together with low blood glucose uptake in tissues, induces a response to metabolic stress (alteration of glucose, glycogen and lipid metabolism) in the liver; this response would promote an increase in oxidative stress associated with high mitochondrial activity, in which ROS levels increase (Shimizu *et al*., 2012; Mohamed *et al*., 2016; Jiang *et al.*, 2020) and consequently lead to an increase in MDA and POx in diabetic rats (Table No. 7); however, antioxidative effect of CEG was stronger in female diabetic rats than male rats, and this discrepancy will need to be studied in greater detail in future research.

All these antioxidant results agree with those reported by Sun *et al.* (2017), where a flavonoid fraction isolated from a *Gnaphalium* extract (150 mg/kg) did not show a hypoglycemic effect even after being administered for 20 days in STZ-induced diabetic mice, nor did it improve their prognosis in an FBG test as was also the case of the rats in our study after administering them for 15 days with the complete extract at a dose close to 200 mg/kg, but it did improve the antioxidant response *in vivo* in the diabetic mice causing an improvement in its prognosis.

In the general context of this study, the function of β cells in the pancreas was inhibited in diabetic rats and did not recover with CGE administration, despite results demonstrating the effectiveness of its antioxidant activity in alleviating oxidative damage caused by alloxan in pancreatic tissue, lack of insulin production favored the development of metabolic stress (Zhao *et al*., 2023), and maintain hyperglycemia in diabetic CGE-treated rats.

CONCLUSION

Chloroformic extract of aerial parts of *Gnaphalium sp* even though did not decrease blood glucose concentration in diabetic rats (hypoglycemic activity), it is proposed that the polyphenolic (flavonoids) and volatile compounds contained in the chloroformic extract of *Gnaphalium sp* exerted an excellent antioxidant activity that decreased oxidative damage in the pancreas and liver of diabetic rats induced by alloxan, therefore, this work, being the first of its kind, proposes “gorbolobo” as an adjuvant agent capable of mitigating the oxidative damage associated with diabetes and the manifestation of its complications.

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