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***Geranium* Species as Antioxidants**

Mirandeli Bautista Ávila,
Juan Antonio Gayosso de Lúcio,
Nancy Vargas Mendoza,
Claudia Velázquez González,
Minarda De la O Arciniega and
Georgina Almaguer Vargas

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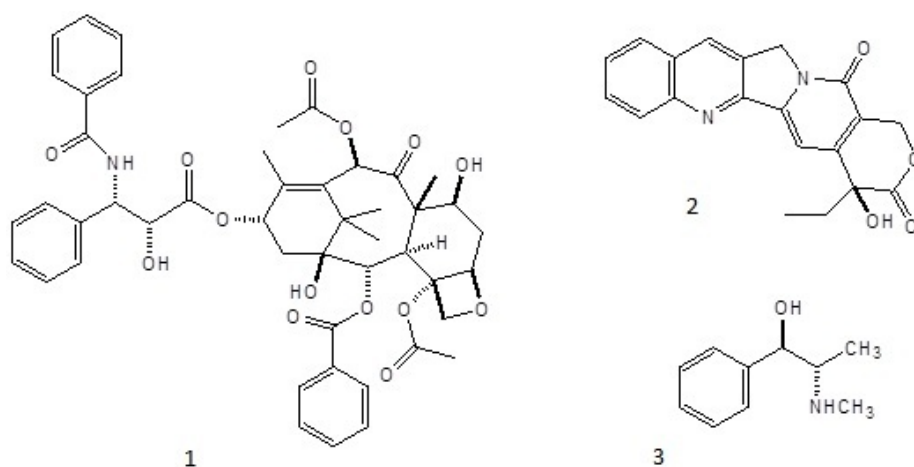
1. Introduction

Complementary alternative medicine (CAM) has been widely used for a long time for the treatment of multiple diseases, despite the great advances in allopathic medicine. It is estimated that about 80% of the world population use some form of CAM.

CAM encompasses empirical knowledge and medical practice in which use is made of herbal medicinal plants, animals, minerals, manual therapy and exercise, alone or in conjunction for the treatment of diseases. In the early 1980's there emerged a strong interest in their study that has significantly influenced the pharmaceutical industry in developing technologies to identify new chemical entities and structures that are used for the synthesis of drugs. It has been shown that natural products play an important role in the discovery of compounds for drug development to treat multiple diseases.

Also, is important to recognize that use plants and their products have provided proven benefits to humanity, which falls into four areas: (i) food, (ii) essences and flavoring agents, (iii) perfumes and cosmetics, and (iv) biological and pharmaceutical agents [1]. Within the pharmaceutical area, the current outlook for natural products in drug discovery takes a central role, since at the beginning of this new millennium, only about 10% of 350,000 known species have been investigated from a phytochemical or pharmacology point of view [1].

A great examples of molecules that have hit the market as drugs by isolation from natural products metabolites are: taxol (1), an antitumor agent isolated from *Taxus* species [2] and camptothecin (2), isolated from the Chinese plant *Camptotheca acuminata* Decne (Nyssaceae), used to treat ovarian, breast and colorectal cancer, another example is ephedrine (3), which is isolated from the plant *Ephedra sinica*³ and is used as a flu remedy. In drug discovery, researchers around the world use plants as an essential route in the search for new drugs leaders. One of the main objectives of the research laboratories is the preliminary meeting with isolated bioactive natural products, and its uses as anti-cancer, antiviral, antimalarial, antifungal and anti-inflammatory [3]. The search for active compounds in plants is an essential way for the development of new drugs, a process in which there is now more advanced and specific methodologies for the analysis of biological activities in particular.



Documentary research from 1981 to 2006 showed that natural products have been a source of 5.7% of drugs produced in those years. The derivatives of natural products are most of the times, chemical molecules synthesized from natural products and contributed to the 27.6% of the total of the new molecule.

2. Characterization of *Geranium* genus

Geranium genus is taxonomically classified within the family *Geraniaceae* Juss, which includes five to eleven genres, and in total near to 750 species. The genus best known are *Geranium* genus, as wild plants (Figure 1) and *Pelargonium* genus, as garden plants. The names of these genres usually cause confusion because "geranium", is the common name for certain species of *Pelargonium*.

The names come from Greek and refer to the form that its fruits acquire, likes beaks. Thus, the word "Geranium" comes from "geranos" meaning crane, and "Pelargonium" derived from "Pelargos" meaning stork [4].



Figure 1. *Geranium* genus.

Subgenus	Section	Number of Species
<i>Erodioidea</i>	<i>Erodioidea</i>	3
	<i>Aculeolata</i>	1
	<i>Subacaulia</i>	15
	<i>Brasiliensia</i>	3
<i>Geranium</i>	<i>Geranium</i>	339
	<i>Dissecta</i>	4
	<i>Tuberosa</i>	19
	<i>Neurophyllodes</i>	6
	<i>Paramensia</i>	2
	<i>Azorelloida</i>	1
	<i>Polyantha</i>	7
	<i>Trilopha</i>	5
	<i>Divaricata</i>	2
	<i>Batrachioidea</i>	4
<i>Robertium</i>	<i>Ungiculata</i>	5
	<i>Lucida</i>	1
	<i>Ruberta</i>	4
	<i>Anemonifolia</i>	2

Table 1. *Geranium* genus clasification

Within the classification of *Geranium* genus are accepted 423 species, distributed in three subgenuses: *Erodioidea*, *Geranium* and *Robertium*. The following table (Table 1) shows the distribution of this classification.

Currently, in Hidalgo state, in Central Mexico, are classified 8 different species [5] and any one has chemical or pharmacological studies.

2.1. Biological activities and compounds isolated from *Geranium* species

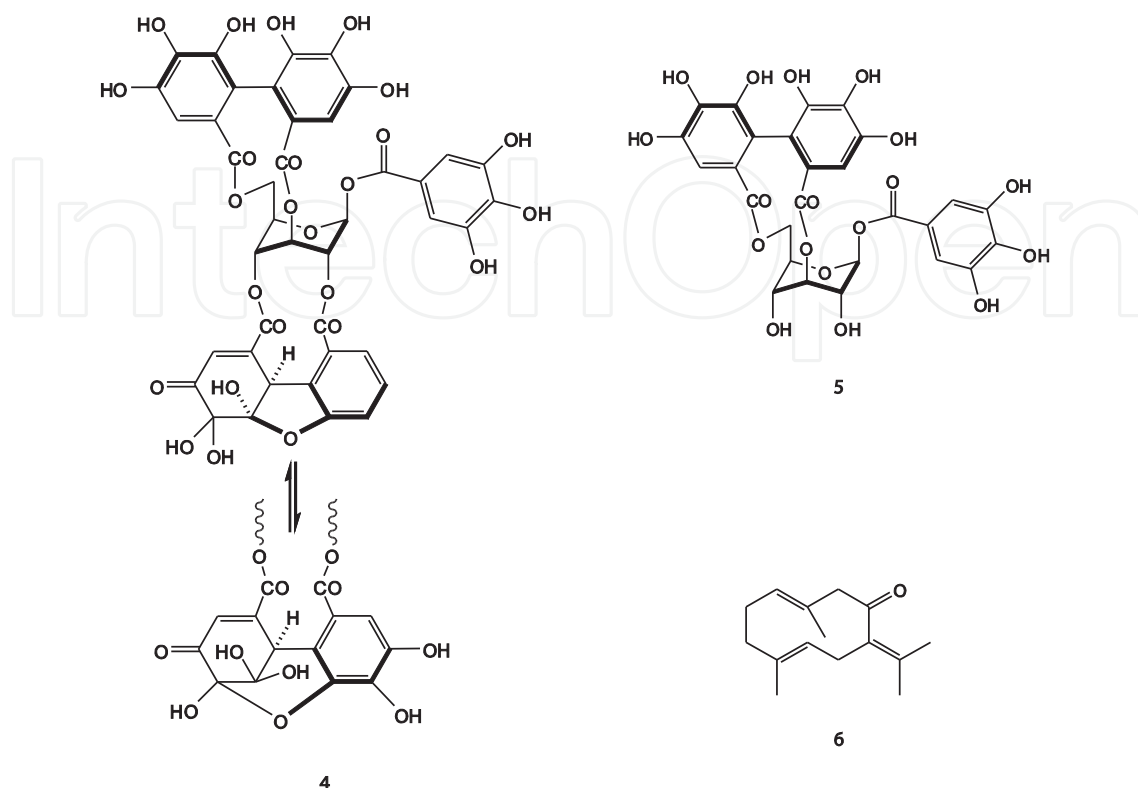
Some species of *Geranium* that have been studied has shown biological activity like: hypotensive, mild astringents, diuretics, hepatoprotection, antioxidants, anti-inflammatory and antiviral. *Geranium* species also are used as a remedy for tonsillitis, cough, whooping cough, urticarial, dysentery, kidney pain and gastrointestinal disorders [6-8]. It is probably that the species of this genus that growing in the State of Hidalgo possess a similar biological activities and metabolites. All phytochemicals studies described for these species, indicates the presence of polyphenolic compounds called tannins, which have been considered as water-soluble compounds of molecular weight between 500 and 30,000 g/mol with special properties such as the ability to precipitate alkaloids, gelatin and other proteins [9]. Nowadays tannins are well known because of its antioxidant properties. Tannin-protein complexes in the gastrointestinal tract provide persistent antioxidant activity.

One of the major components in *Geranium* species isgeraniin (4) [10] described by its discoverer as a crystallizable tannin. This substance first isolated from *Geranium thunbergi* Sieg. Et Zucc. by T. Okuda in 1976, has been evaluated showing an antihypertensive activity, geraniin inhibits the angiotensin converting enzyme [11,12] and reverse transcriptase of tumoral viruses RNA [13], inhibit HSV-1 and HSV-2 multiplication at different magnitudes of potency and also is an excellent antioxidant [14]. The corilagin (5) [15] is a derivative of geraniin, which has presented antimicrobial activity among other activities [16].

2.2. Different species of geraniums and its relevant compounds

The specie *Geranium macrorizum* presented a significant hypotensive activity in anesthetized cats [17], plus antioxidant activity. Of this specie germacrone (6) was isolated which is considered a precursor of pheromones.

Geranium robertianum L. well-known specie and one of the most variable in Britain has been used in conditions where increased diuresis is required, such as cystitis, urethritis, pyelonephritis, gout, hypertension and edema. Nowadays the phytochemistry of this geranium is relatively well known and its most studied active compounds are tannins, volatile oils, flavonoids and polyphenols (hyperoside, ellagic acid, isoquercitrin, quercitrin, kaempferols, caftaric acid, rutoside). Also infusions and decoctions prepared from leaves of this geranium: Robert herb or red Robin, are described as anti-hyperglycaemiant and commonly used in Portuguese herbal medicine [18]. In other hand *G. robertianum* extract treatment increased the efficiency of coupling between oxidative and phosphorylative systems, since RCR was considerably higher in GK rats consuming this plant extract [19].

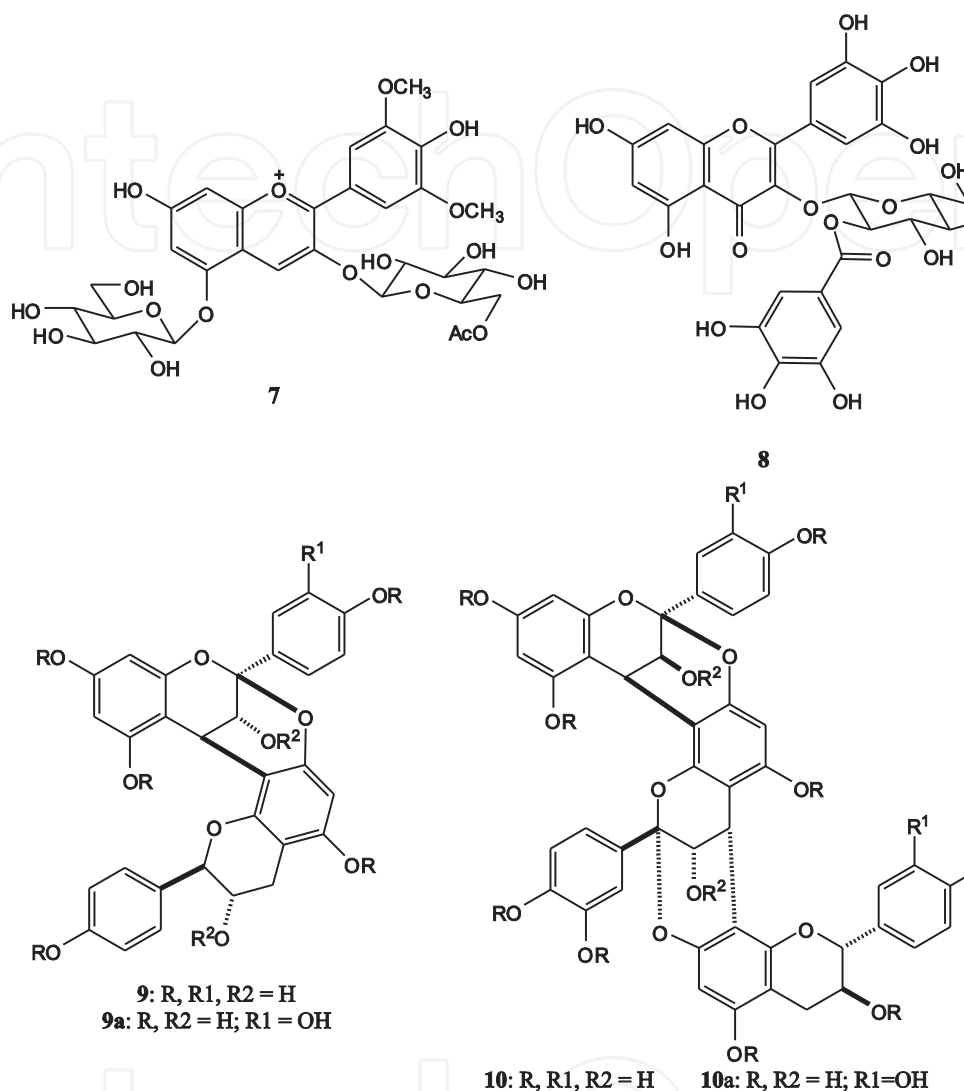


Recently the extracts of *G. sylvaticum* were studied [20] for antioxidant potential and all tested extracts had strong antioxidant activity and will be subject for further investigations. From flowers of *Geranium sylvaticum* was isolated 3-O-(6-O-acetyl- β -D-glucopyranoside)-5-O- β -D-glucopyranoside of malvidin (7) [21]. *Geranium sanguineum* L. showed significant inhibitory activity of influenza virus and herpes simplex (8). The methanolic extract of *Geranium pratense* inhibited the action of the amylase enzyme in mouse plasma, isolated for first time the 3-O-(2-O-galloyl) - β -D-glucopyranoside myricetin(9) [22].

Geranium niveum, widely used by the Tarahumara Indians of Mexico. Is a specie rich in proanthocyanidins and other phenolics [23]. Previous in vitro assays have demonstrated that proanthocyanidins exhibited antiinflammatory, antiviral, antibacterial, enzyme-inhibiting, antioxidant, and radical-scavenging properties, the roots of this species were isolated new proanthocyanidins named as geraniins A (9) and B (9a), latterly in 2001 were found geraniins C (10) and D (10a) [24]. A recently study showed that geraniin A has antioxidant activity [25].

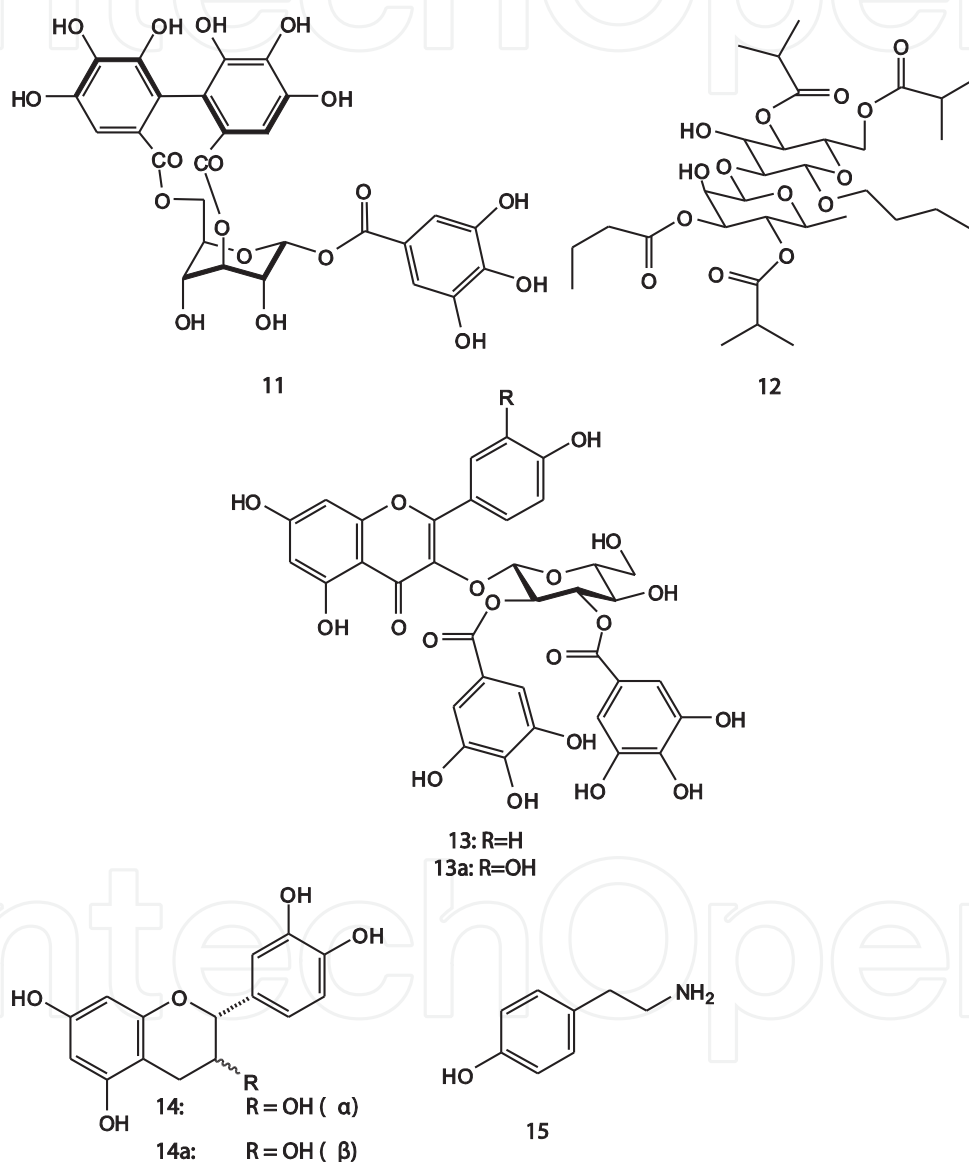
Geranium pusillum, commonly known as Small-flowered Cranesbill or (in North America) small Geranium, contains 1-O-galloyl-3,6-hexahydroxibifenil-D-galactopyranoside (11) (pusilagin) a polyphenolic compound extracted from aerial parts [26]. The aqueous ethanolic extract of *Geranium wallichianum* showed antibacterial activity against *Staphylococcus aureus* [27] and the study of the chemical constituents of the whole plant has resulted in the isolation and characterization of six compounds. These six compounds were identified as ursolic

acid, β -sitosterol, stigmasterol, β -sitosterol galactoside, herniarin, and 2,4,6-trihydroxyethylbenzoate which were isolated for the first time from *Geranium wallichianum* [28].



Geranium caespitosum produces neohesperidoside (12) able to potentiate 10 to 100 times the action of drugs such as ciprofloxacin, norfloxacin, berberine and rhein, against bacterias such as *S. aureus*, *NorA S. aureus*, *B. and B. megaterium subtilis* [29]. Besides, *Geranium carolinianum* L., is a commonly used traditional Chinese medicine (TCM) with the efficacy of eliminating wind-damp and treating diarrhea. It is clinically used to treat the arthralgia due to wind-dampness, anaesthetization and muscular constriction. It has been reports that *Geranium carolinianum* L. as well of most of the congeneric plants contain significant amounts of tannins, flavonoids, organic acids, and volatile oils [30]. Also, has shown that roots contain a substance that is extracted with water and can be a biological mechanism to control bacteria (*Ralstonia solanacearum*) which attacks potatoes [31].

From *Geranium pyrenaicum*, which showed antileishmanial activity [32], a new glycosylate flavonoid: 3-O-(2'', 3''-di-O-galloyl)- β -D-glucopyranoside of kaempferol (13) was isolated, and an uncommon quercetin derivative: 3-O-(2'', 3''-di-O-galloyl)- β -D-glucopyranoside of quercetin (13a) too. In *Geranium mexicanum* an antiprotozoal activity was assayed from its roots, where the most active compound founded was the flavan-3-ol-(-)-epicatechin (14), showing moderate activity (+)-catechin (14a), tyramine (15) and 3-O- β -D-glucopyranoside of β -sitosterol [33].



Geranium bellum Rose is a perennial plant with long roots, found in the grassy meadows bordering pine/oak forests in the mountains of Hidalgo State, Mexico, where it has the popular name “pata de león” and has been used as a traditional remedy for treatment of fevers, pain, and gastrointestinal disorders. Radical scavenging assay-guided fractionation of the antioxidant EtOAc and MeOH extracts from the aerial parts of *Geranium bellum* resulted in the isolation of b-

sitosterol 3-O- β -D-glucopyranoside, quercetin 3-O- α -L-(2''-O-acetyl)arabinofuranoside (16), quercetin 3-O- α -L-arabinofuranoside, quercetin, methyl gallate, gallic acid, methyl brevifolin carboxylate (17), and dehydrochebulic acid trimethyl ester (18). Compounds 2, 7 and 8 are isolated for the first time from *Geranium* genus [34]. Antioxidant activity of these extracts (both initial fractions and pure compounds), was tested by measuring their capacity to scavenge 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals, an assay widely used for screening of antioxidant activity of natural products [15].

Constituents from the aerial parts of *Geranium potentillaefolium* founded in certain studies were geraniin, corilagin, gallic acid, methyl gallate, methyl brevifolincarboxylate, quercetin, quercetin 3-O- β -D-glucopyranoside, quercetin 3-O- β -D- [6''-O-galloyl]glucopyranoside, kaempferol, β -sitosterol 3-O- β -D-glucopyranoside and β -sitosterol [35].

3. Study of *Geranium schiedeanum*

Geranium schiedeanum (Gs) (Figure 2), species that grows in Central Mexico, has been used as an antipyretic, anti-inflammatory and antiseptic. The use of other geranium species also has been reported a hypoglycemic, antihypertensive and cholesterol-lowering effect. However, scientific evidence does not exist in any literature to corroborate these targets or any other. In the present study the effect of Gs were studied in reference to postnecrotic liver damage induced by thioacetamide (TA).



Figure 2. *Geranium schiedeanum* [5]

3.1. Plant material

Specimen of *Geranium schiedeanum* was collected at Epazoyucan Municipality, in Hidalgo State, México, during June 2009. A voucher specimen (J. A. Gayosoo-de-Lucio) is preserved at the Herbarium of Biological Research Centre, Autonomous University of Hidalgo, Pachuca, Hidalgo, Mexico where Professor Manuel González Ledesma identified the plant material.

3.2. Extraction and purification

Air-dried aerial parts (1 kg) were extracted acetone-H₂O 7:3 (20 L) by maceration for 7 days. Vacuum evaporation of dissolvent give a 5 L residue Filtration give a fatty solid residue (12g) and complete evaporation of water give the acetone-water extract (115 g).

Lots of 3 g of acetone-water extract were purified on a Sephadex LH-20 (25 g) column using H₂O, H₂O-MeOH (9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4, 1:9) and MeOH, as eluents. Fractions of 300 mL of each polarity were collected and marked "A-K". They were evaporated and analyzed by TLC and NMR. Fractions "B" gave 75 mg, and were purified over silica gel (10 g), using CHCl₃, CHCl₃-AcOEt(9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4, 1:9) and AcOEt (10 mL of each polarity), as eluents and collecting fractions of 7 mL, fractions 13-16 give I 25 mg. Fractions "C" and "D" gave 56 mg, and were purified over silica gel (10 g), using CHCl₃-MeOH (50:7.0, 48:7, 45:7, 40:7, 35:7 and 30:7, 40 mL of each), as eluents and collecting fractions of 7 mL, fractions 33-66 give II 2 mg, (these procedure was repeated ten times to obtain 18 mg of compound), Fractions "F-I" gave 1.8 g, a portion of 500 mg were purified over silica gel C-18 (5 g) using H₂O, H₂O-MeOH (9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4, 1:9) and MeOH (20 mL of each polarity), fractions of 10 mL were collected fractions 2-4 gave 325 mg of III, fraction "K" gave 90 mg were added 5 mL of (40°C) pyridine and were placed a room temperature for 72 h, filtrated of mixture give a yellow needles 60 mg of IV (Figure 3).

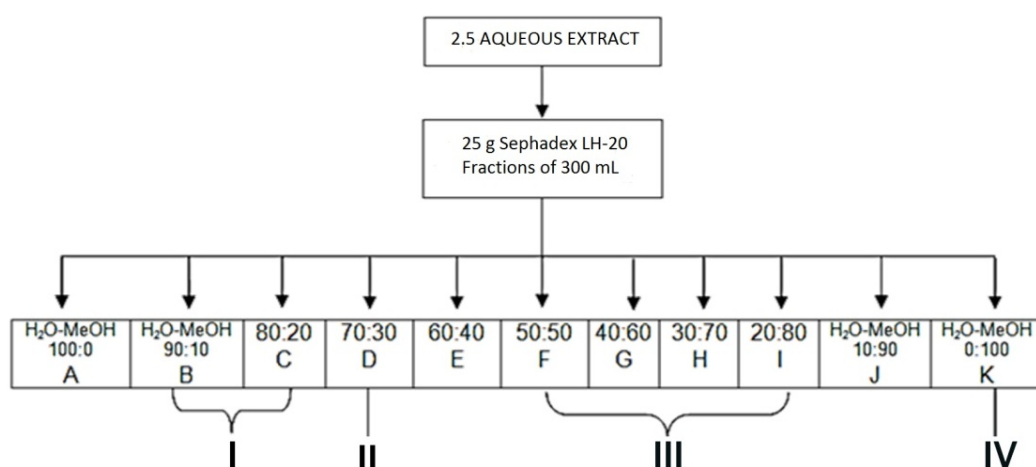


Figure 3. Extract fractions scheme.

3.3. Animals and treatment

Male adult Wistar rats 2 months old (200–220g) were obtained from UAEH Bioterio, and acclimated to our animal room for two weeks before use. Throughout these two weeks rats were supplied with food and water *ad libitum*, exposed to a 12 h light-dark cycle and given intraperitoneally a single necrogenic dose of thioacetamide (6.6 mmol/Kg body weights) freshly dissolved in 0.9% NaCl. The dose of thioacetamide was chosen as the highest dose with survival above 90% [36,37]. Wistar rats were intragastric pre-treated or not with a single dose of Gs extract (300 mg/kg) during 4 days, the fourth day of pretreatment were intraperitoneally injected with a single dose of TA (6.6 mmol/Kg). Samples of blood and liver were obtained from rats at 0, 24, 48, 72 and 96 h following TA intoxication. Untreated animals received 0.5 ml of 0.9% NaCl. Experiments were performed on two different groups: rats treated with a single dose of thioacetamide (TA) and rats pre-treated with Gs and treated with a single dose of thioacetamide (Gs + TA). Each experiment was performed in duplicate from four different animals and followed the international criteria for the use and care of experimental animals outlined in *The Guiding Principles in the use of Animals in Toxicology* adopted by the Society of Toxicology in 1989.

3.4. Processing of samples

In order to clarify the sequential changes during the different stages of liver injury and the post-necrotic regenerative response, samples were obtained from control and at 24 and 48 h of TA intoxication in both Gs pre-treated or non pre-treated animals. Rats were sacrificed by cervical dislocation and samples of liver were obtained and processed as previously described. Blood was collected from hearts and kept at 4 °C for 24 h, centrifuged at 3000 rpm for 15 min, and serum was obtained as the supernatant.

3.5. Determination of AST

Enzymatic determination were carried out in serum in optimal conditions of temperature and substrate and cofactor concentrations. Aspartate aminotransferase (AST) activity were determined in serum. AST (EC 2.6.2.1) and was assayed following the method of Rej and Horder [38].

The activity of this enzyme was determined spectrophotometrically, by measuring the decrease in absorbance at 340 nm at 37 °C, produced by the oxidation of NADH to NAD⁺ in the coupled reaction of reduction of oxaloacetate to malate, catalyzed by malate dehydrogenase, according to the following process:

3.6. General

IR spectra measured in MeOH on a Perkin Elmer 2000 FT-IR spectrophotometer. Optical rotations were determined in MeOH on a Perkin Elmer 341 polarimeter. NMR measurements performed at 400 MHz for ¹H and 100 MHz for ¹³C on a VARIAN 400 spectrometer from CDCl₃, CD₃OH, DMSO-d₆ solutions. Column chromatography (CC) was

carried out on Merck silica gel 60 (Aldrich, 230-400 mesh ASTM) and sephadex LH-20 Sigma Aldrich.

3.7. Statistical analysis

The results were calculated as the means \pm SD of four experimental observations in duplicate (four animals). Differences between groups were analyzed by an ANOVA following Snedecor F ($\alpha = 0.05$). Students' test was performed for statistical evaluation as follows: (a) all values against their control; b) differences between two groups Gs + TA versus TA.

3.8. Results

3.8.1. Active compounds of *Geranium schiedeanum*

One kg of the aerial part of *G. schiedeanum* was extracted by maceration for 7 days with 20 L acetone-water (7:3), concentrated under reduced pressure to a volume of 3 L, which was extracted with CHCl_3 yielding 12.75 g of CHCl_3 phase and 125 g of aqueous phase. The phytochemical study of *Geranium schiedeanum* led to the isolation of hydrolysable tannins (I) gallic acid, (III) acetylgeraniin and (IV) ellagic acid and to a lesser proportion of kaempferol glycosideflavonoid (II) (Figure 4 and 5). Is relevant to notice that is the first time discloses the compound II in the *Geranium* genus and further that the yield of compound III in the crude extract was 40%.

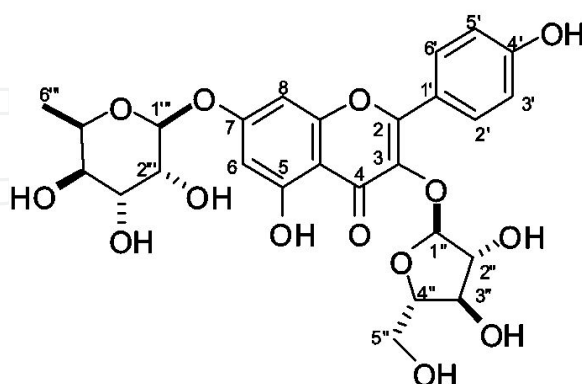


Figure 4. Compound (II) 3-O- α -L-arabinofuranoside-7-O- β -L-rhamnopyranoside de Kaempferol

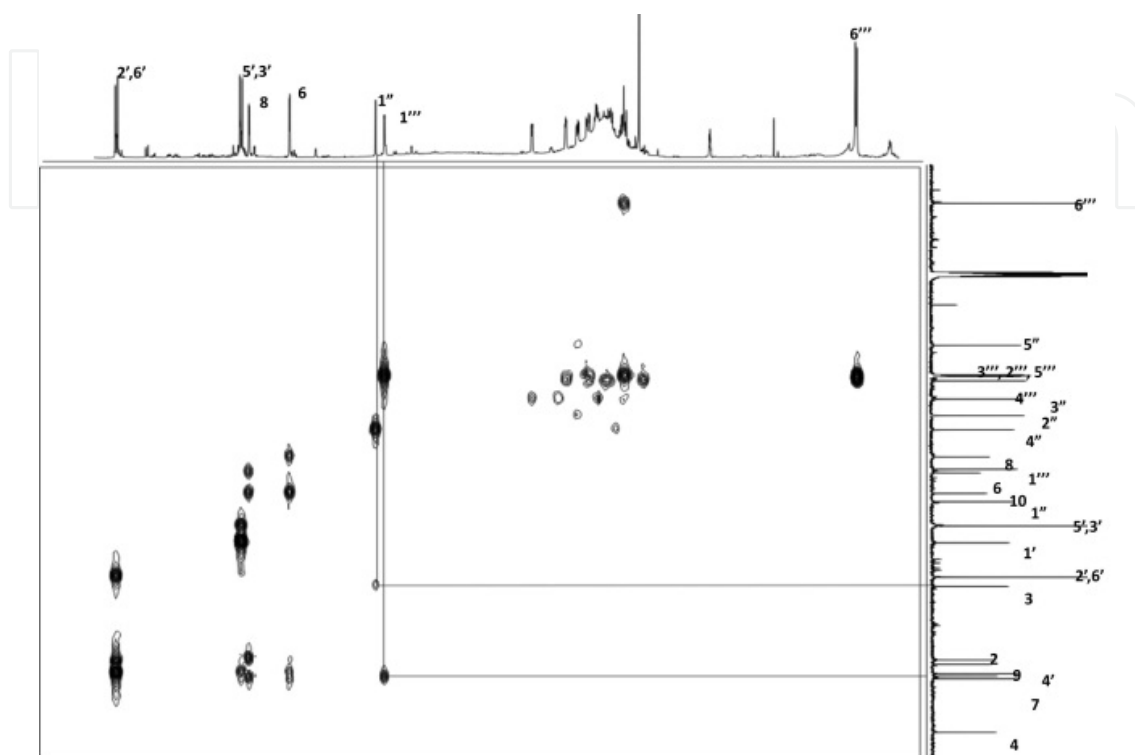


Figure 5. HMBC experiment of compound II

3.8.2. Aspartate aminotransferase

The acute liver injury induced by a necrogenic dose of thioacetamide (TA), a potent hepatotoxic agent, is characterized by a severe perivenous necrosis [39]. The necrosis develops as a consequence of the biotransformation of TA through the microsomal flavin-dependent monooxygenase [40]. The reactive metabolites responsible for TA hepatotoxicity are the radicals derived from thioacetamide-S-oxide and the reactive oxygen species derived as sub products in the process of microsomal TA oxidation, both of which can deplete reduced glutathione leading to oxidative stress [41, 42].

Liver damage induced by xenobiotics is characterized by the release in serum of hepatic enzymes due to necrosis of hepatocytes. AST is randomly distributed in the hepatic acinus, and is the enzyme activity used as marker of necrosis. Our results showed that Gs extract significantly reduced the level of liver injury. The levels of AST (Figure 6) were significantly lower in the rats pretreated with Gs.

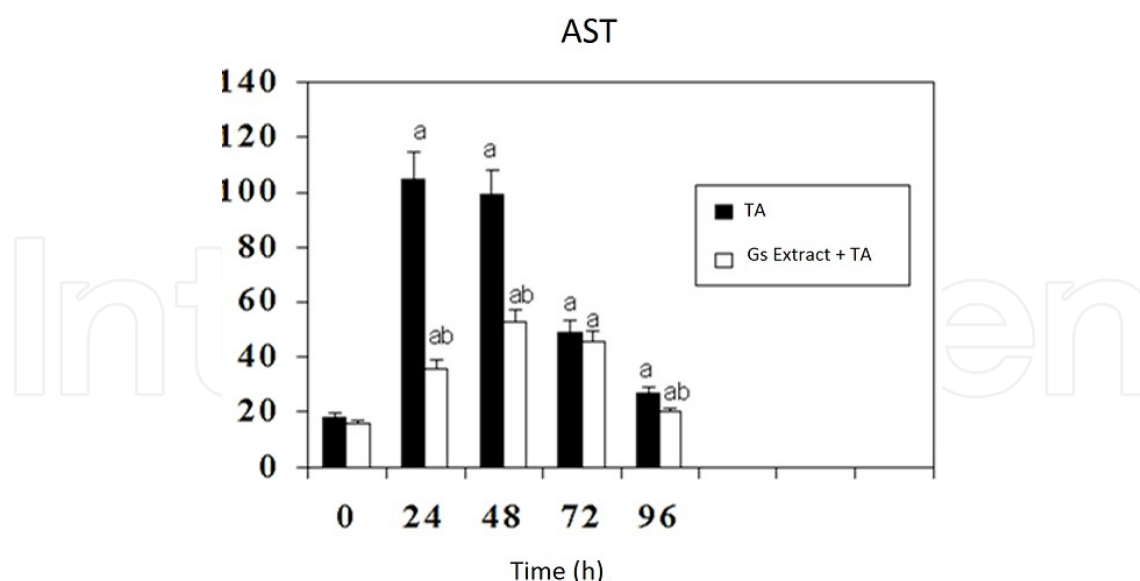


Figure 6. Effect of Gs pre-treatment on aspartate aminotransferase activity in serum of rats intoxicated with one sub-lethal dose of thioacetamide. Samples were obtained at 0, 24, 48, 72 & 96 h following thioacetamide (TA). The results, expressed as nmol per min per ml of serum, are the mean \pm SD of four determinations in duplicate from four rats. Differences against the respective control are expressed as (a) and differences due to Gs extract are expressed as (b) $p < 0.05$.

4. Conclusion

There is evidence that free radicals play a critical role in certain pathological conditions such as some cancers, multiple sclerosis, inflammation, arthritis and arterosclerosis [43]. For this reason, some research objectives directed toward the development or discovery of these compounds catchers of these radicals.

A large number of plant species, like *G. schiedeanum* contain chemical compounds that exhibit the ability to trap free radicals. The ability to trap free radicals has been called antioxidant activity. The phytochemical study of *Geranium shiedeanum* led to the isolation of hydrolysable tannins, well known as potent antioxidants: gallic acid, acetonylgeraniin and ellagic acid and a lesser proportion of kaempferol glycoside flavonoid (3-O- α -L-arabinofuranoside-7-O- β -D-rhamnoside de Kaempferol), notably is the first time discloses these compounds in the genus. Further the yield of acetonyl geraniin in the crude extract was 40%.

Also, in the present study TA-induced hepatotoxicity was used to investigate the effect of the pretreatment of *G. schiedeanum* on the events involved in liver regeneration. The results obtained in the present study provide evidence that Gs, when administered intravenously prior to TA, significantly reduce liver damage.

The pre-treatment with the crude extract in the model of thioacetamide-induced hepatotoxicity in rats, decreased and delayed liver injury by 66% at 24 h.

The data obtained indicate that the crude Gs extract pre-treatment has hepatoprotective and antioxidant effect in damage induced by TA. This result suggests that Gs extract may be used as an alternative for reduction of liver damage. However further investigation on the acute toxicity and on the mechanism of the hepatoprotective effect of the plant species needs to be carried out.

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Author details

Mirandeli Bautista Ávila, Juan Antonio Gayosso de Lúcio, Nancy Vargas Mendoza, Claudia Velázquez González, Minarda De la O Arciniega and Georgina Almaguer Vargas

Universidad Autónoma del Estado de Hidalgo, Mexico

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