CHOLERETIC AND ANTIOXIDANT EFFECT OF *EUPATORIUM BUNIIFOLIUM.*

Gorzalczany S[1], Muschietti L[2], Repetto M[3], Kartin L[4], Ferraro G. [2], Martino V [2], Acevedo C. [1]

[1]Cátedra de Farmacología, [2]Cátedra de Farmacognosia, IQUIMEFA (UBA-CONICET) [3] Cátedra de Química General Inorgánica, [4] Cátedra de Química Analítica Instrumental

Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (1113), Buenos Aires, Argentina.
Corresponding author: sgorza@ffyb.uba.ar

Summary

Eupatorium buniifolium is widely distributed in Argentina and it is used in folk medicine as digestive and in hepato-biliary complaints. The aqueous extract of the aerial parts has been tested in rats for its choleretic, antioxidant activity and acute toxicity effects. The extract showed significant choleretic effect (35%, i.v. and 50%, p.o.) after both oral (500 mg/kg) and intravenous (250 mg/kg) administration without changes in the bile acids output. Measure of Total Reactive Antioxidant Potential (TRAP) and the Total Antioxidant Reactivity (TAR) indicate that the extract has antioxidants of high reactivity antioxidant capacity. It may be due to the presence of antioxidants such as phenolic and flavonoid compound, extensively reported as free radicals scavengers. Toxicity signs were not observed until oral dose of 3000 mg/kg.

Keywords: *Eupatorium buniifolium*, choleretic, antioxidant, acute toxicity, aqueous extract, flavonoids.

Higher plants used in ethnomedicine can provide important lead compounds in modern drug development.
However, the search of pure active principles is time consuming and expensive. A standardised active extract or a standardised fraction may provide effective herbal formulations.
Eupatorium buniifolium (EB) Hook. et Arn., (*Asteraceae*),) commonly known as "romerillo", "romerillo colorado" or "chilca", is widely distributed in Northeastern and Central Argentina. This species has been used in folk medicine as antirrheumatic agent (1), disinfectant (2), digestive and for the treatment of liver, kidney and nervous system diseases (3).
Previously we reported pharmacological activities as, antiviral (4), anti-inflammatory and analgesic activity (5,6) and recently we also reported depressant effect (7). In the other hand, phytochemical research on the aerial parts has revealed the presence of flavonoids, triterpenoids, hydroxycinnamic acids and derivatives (8, 9,10). Many plants are used in traditional medicine as "digestive" and this term may include several activities. In this sense no scientific evaluation of the main ethnomedical uses of EB appears to have been undertaken so far.
Taking into account these facts, the aim of this study was to evaluate the choleretic, antioxidant activities and acute toxicity of the aqueous extract of EB (AEEB) in order to validate the traditional uses as digestive and the treatment of hepatic troubles (3) and establish its safety. Moreover, the total polyphenol and flavonoid content was determined.

Materials and methods

Drugs

Luminol, 2,2'-azo-*bis*(2-amidinopropane) (Dorwill, Argentina), Trolox and Sodium dehidrocholate were obtained from Sigma, USA. All of the other reagents were used of analitycal grade.

Plant material

Aerial parts of *E. buniifolium* were collected in the Province of Entre Ríos, Argentina and identified by Ing. Juan de Dios Muñoz. A voucher specimen (Nº 5067) has been deposited at the Herbarium of the Museo de Farmacobotánica of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires.

Extraction procedment

25 g of the aerial parts of EB air-dried and ground to powder were extracted with 500 ml of water at 100ºC for 20 min., the filtrate was lyophilised to provide a yellowish powder (yield: 8% P/P).

Animals

Female Sprague Dawley rats (200-250 g) and CF1 mice (18-23 g) of both sexes were used for choleretic activity and acute toxicity assay respectively, according to international principles and local regulations concerning the care and use of laboratory animals (11). The animals had free access to a standard commercial diet and water *ad libitum* and were kept in rooms with a controlled 12 / 12 hours light / dark cycle (8.00 a.m. to 8.00 p.m.) and temperature (22º ± 2º C).

Acute toxicity

Groups of 10 CF-1 mice, 5 male and 5 female, were used. Food was withdrawn 16 h before oral administration of single doses of the extract. The control group received only vehicle (water), and the remaining groups received increasing doses up 3 g/kg (0.2 ml/10 g body weight) of AEEB orally, by means of a gastric catheter. The animals were maintained in a cage with free access to a standard diet and water *ad libitum.* After a single administration, signs of possible toxicity were evaluated every hour for the first 6 h and twice a day for up to 15 consecutive days. Surviving animals were weighed daily (12, 13). On the fifteenth day, animals were sacrifice using high dose anaesthesia (thiopental sodium, 50 mg7kg) and vital organs, such as lungs, liver, hearth, stomach were observed.

Choleretic activity assay

Rats were starved for 18 h before the experiment with free access to water. Animals were anesthetized with urethane (1.2 g/kg i.p.). The abdomen was opened by a midline incision and the common bile duct was exposed and cannulated just before the hepatic hilus in order to avoid contamination with pancreatic juice. Rectal temperature was monitored and maintained at 37±0.5°C throughout the experiment, using a warming lamp. Bile was collected by gravity in pretared vials at 15 min. intervals for 120 min (14). Bile flow was determined by weight assuming that the specific gravity of rat bile is 1.0 and was expressed as µl/ min/kg body weight.

For oral administration, four groups of five animals were treated by gastric gavage with AEEB dissolved in water at doses of 250 and 500 mg/kg (10 ml/Kg), sodium dehidrocholate (DHC) 500 mg/kg (Reference group). Control animals received the same volume of water (10 ml/kg). Inmediately the animals were anesthetized with urethane (1.2 g/kg i.p.) and the biliar fistula were made and the bile was collected in order to determinate the bile flow. For i.v. administration, another five groups of five rats each were treated with AEEB dissolved in saline solution at the doses of 125, 250 and 500 mg/kg i.v (0.1 ml/100g) (femoral vein). Reference groups received sodium dehydrocolate (DHC), 20 mg/kg i.v. Control animals received a similar volume of saline solution 0.1 ml/100g. After a constant basal flow, bile was collected during 15 min (basal value). Every 15 min after AEEB, DHC or saline injection and during 120 min, variation of basal bile flow (BF), for each animal was calculated using the following formula: (BF – Basal BF)/Basal BF x 100 = %BF

Quantitative determination of bile acids

Quantitative determination of the major conjugated and free 3-hydroxy bile acids in rat bile was done by using a micromethod consisting on a NAD-linked 3 hydroxysteroid dehydrogenase reaction with spectophotometric determination of the resultant NADH, which is an indirect measure of the total bile acids present in the sample (15).

Antioxidant Activity

Ten milligrams of dried extract were diluted in 1 ml of distilled water and diluted solutions were prepared to measure the Total Reactive Antioxidant Potential (TRAP) and the Total Antioxidant Reactivity (TAR) by luminol-enhanced chemiluminiscence (16).
In order to determinate the TRAP value the reaction medium was prepared with phosphate buffer 100 mM (pH 7.4), 20 mM ABAP, 20 μM luminol and increasing volumes of plant extract. Incubation of the mixture at room temperature generated a nearly constant light intensity that was measured directly in a Packard *tri*-Carb scintillation counter with the circuit coincidence out of mode.
The addition of extract decreases chemiluminescence to basal levels for a period (induction time) proportional to the concentration of antioxidant until luminol radicals are regenerated.
The system was calibrated using vitamin E analog Trolox (150 μM). A comparison of the induction time after addition of known concentrations of Trolox and plant extract allows obtaining. TRAP value as equivalents of Trolox concentration necessary to suppress the emitted luminescence.
TAR index was obtained from the instantaneous decrease in luminescence associated to the extract incorporation in a medium of reaction consistent on 2mM ABAP and 10 μM luminol in phosphate buffer 0.1 M, pH 7,40. The system was calibrated with 40 nM Trolox and the chemiluminiscence was measured in a Packard *tri*-Carb scintillation counter with the circuit coincidence out of mode. Iº/I of the plant extracts and Trolox were compared estimating the TAR index, where Iº and I are the luminiscence intensity before and after the incorporation of the scavenger.

Quantitative determination of polyphenols and flavonoids

The polyphenol contents were measured spectrophotometric method at 750 nm, using pyrogallol as reference standard. This method is based on the formation of blue reaction products by redox reaction with Folin reagent (phosphotungstic acid solution) (17)

Flavonoid contents were determined spectrophotometrically, measuring the flavonoids in AlCl3-complex form of purified ethyl acetate phase obtained after acid hydrolysis. Glycosides and aglycones were determined together in aglycone form (18)

Statistical analysis

The results were expressed as means ± SEM. Differences between control and treated groups were tested for significance using one-way analysis of variance (ANOVA) followed by Dunnett's test, taking $p<0.05$ as significant (GraphPad InStat). (Statistica Program v 5.0 – Stat Soft, Inc.).

Results

Choleretic activity

The effects AEEB administered at different doses i.v on the bile flow are shown in Fig. 1.
Control group presented a slight and regular decrease in bile flow level during the whole experiment. The group administered with the reference drug DHC (20 mg/kg) induced a marked stimulation of BF within the first 15 min., however, this effect was short acting and decreased in the following 30 min. A dose-dependent increase was obtained with AEEB administered at 125, 250 and 500 mg/kg i.v., and was significant since 15 min. (250 mg/kg) after administration and persisted during 120 min. The extract also showed choleretic effect when it was administered orally at dose of 250 and 500 mg/Kg (Fig. 2).

Total bile acids were determined in bile samples obtained within the first 15 minutes, but no changes in the concentration (Control: 23.5 ± 0.81 mM, AE 500 mg/kg: 21.7 ± 3.9mM; AE 750 mg/kg: 21.9 ± 1.6 mM) nor in bile acid output were observed (Control: 169.3 ± 19.5 nmoles/min/100g; AE 500 mg/kg: 244.6 ± 63.4 nmoles/min/100g; AE 750 mg/kg: 177.8 ± 24.0 nmoles/min/100g).

Antioxidant Activity

In order to determine the TRAP and TAR indices of AEEB, a method based on luminol-enhanced chemiluminiscence was used, which is based on the measurement of induction times in the oxidation of ABAP, a free radical source. This method is based on the trapping of peroxyl radicals ($ROO^{\cdot}$), and is capable of detecting most of the significant compounds with antioxidant activity present in complex mixtures of antioxidants such as plant extracts. Since TRAP measurements indicate the quantity of antioxidants present in the plant extract, the TAR was also determined in order to measure the quality (given by the reactivity) of the extract, which showed antioxidant activity.

The TRAP value for the extract obtained from the quenching of luminol enhanced chemiluminiscence was 165 μM Trolox. (dilution 1/100) (Fig.3). The TAR value for the extract diluted 1/100 was 167 μM Trolox. The results indicated that, as described for antioxidant capacity of blood plasma (16), TRAP value is near to TAR value. This may be due to the presence of antioxidants of high reactivity. The chemiluminiscence decay following the plant extract addition is qualitatively different to that obtained employing Trolox.

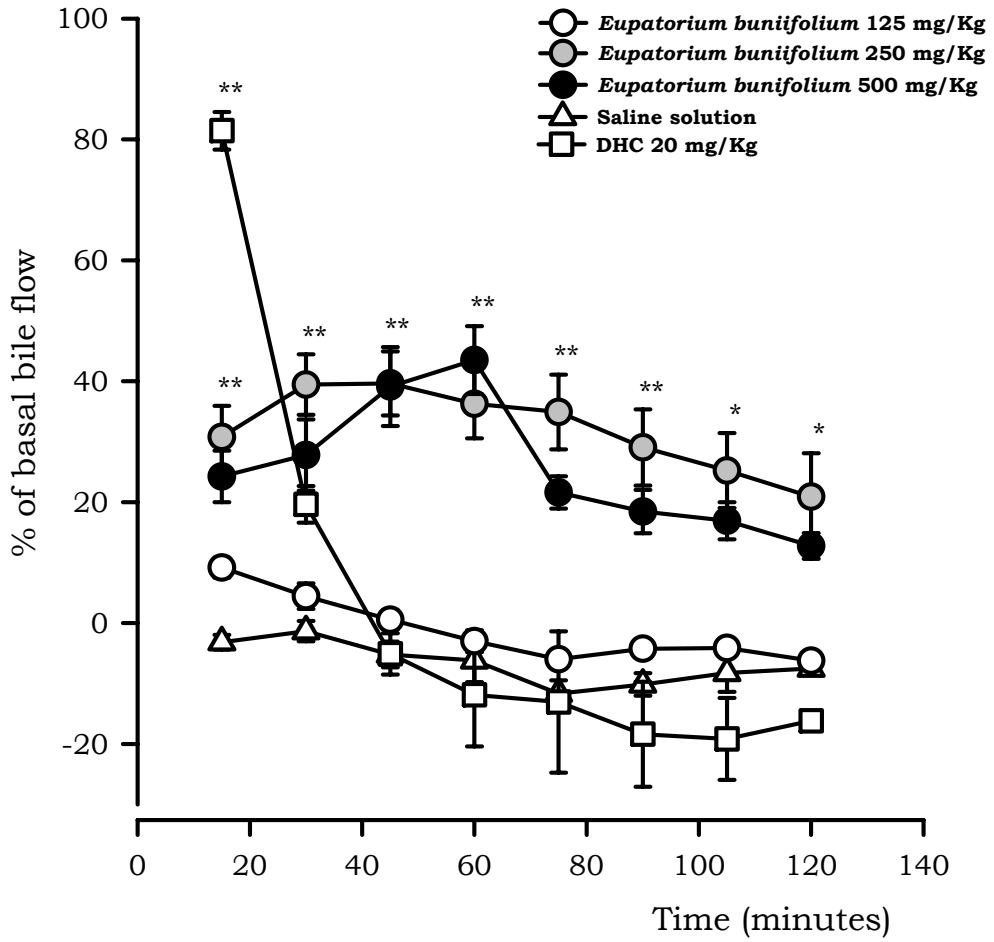

Figure 1. Effect of *Eupatorium buniifolium* aqueous extract (125, 250 and 500 mg/kg i.v.), DHC (20 mg/Kg) and saline solution (control) on bile flow in the rat. Results are expressed as means ± SEM (n=6), *p<0.05, ** p<0.01 versus control.

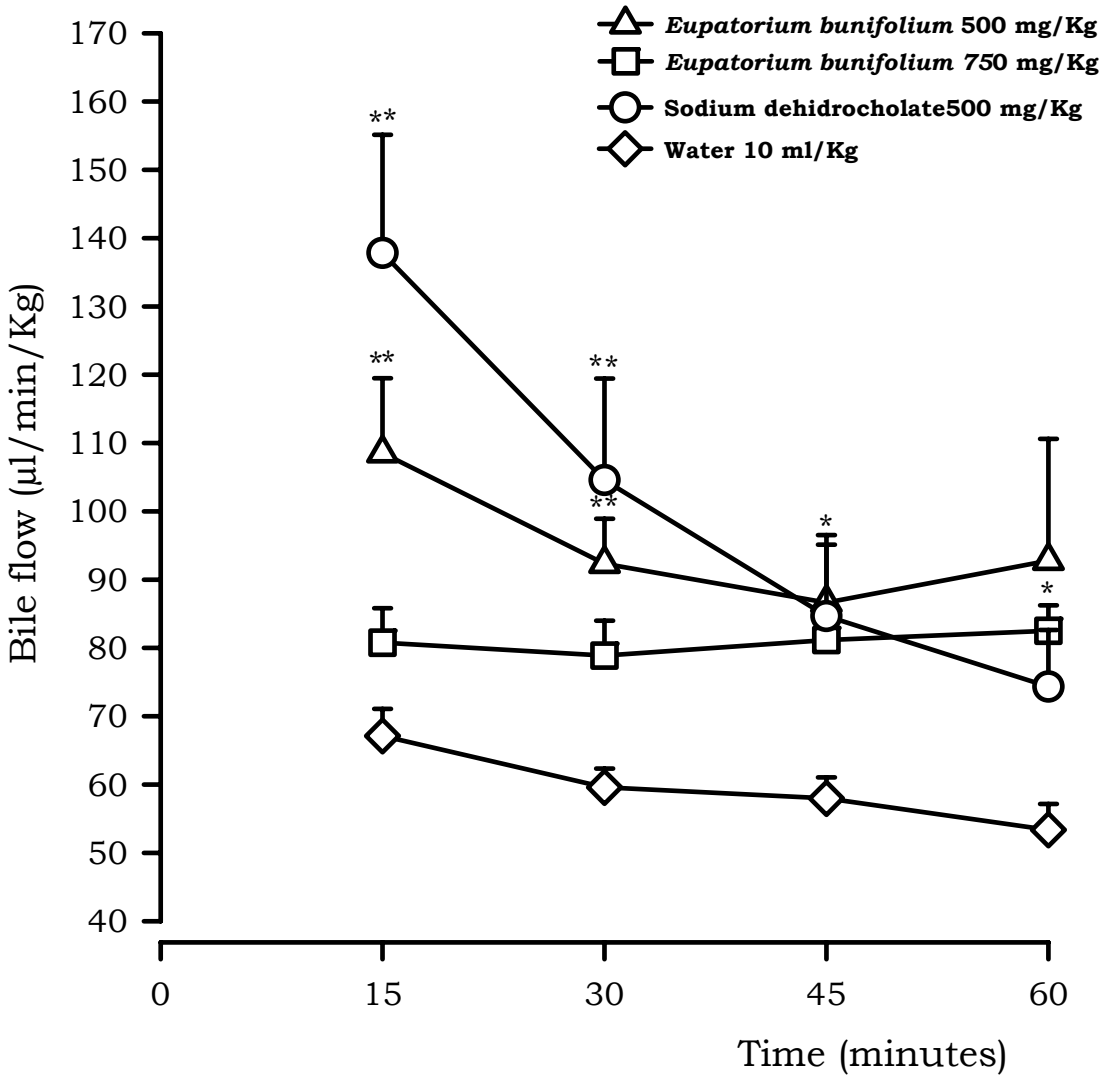

Figure 2. Effect of *Eupatorium buniifolium* aqueous extract (500 and 750 mg/kg p.o.), DHC (500 mg/Kg) and water (control) on bile flow in rats. Results are expressed as means ± SEM (n=6) *p<0.05, ** p<0.01 versus control.

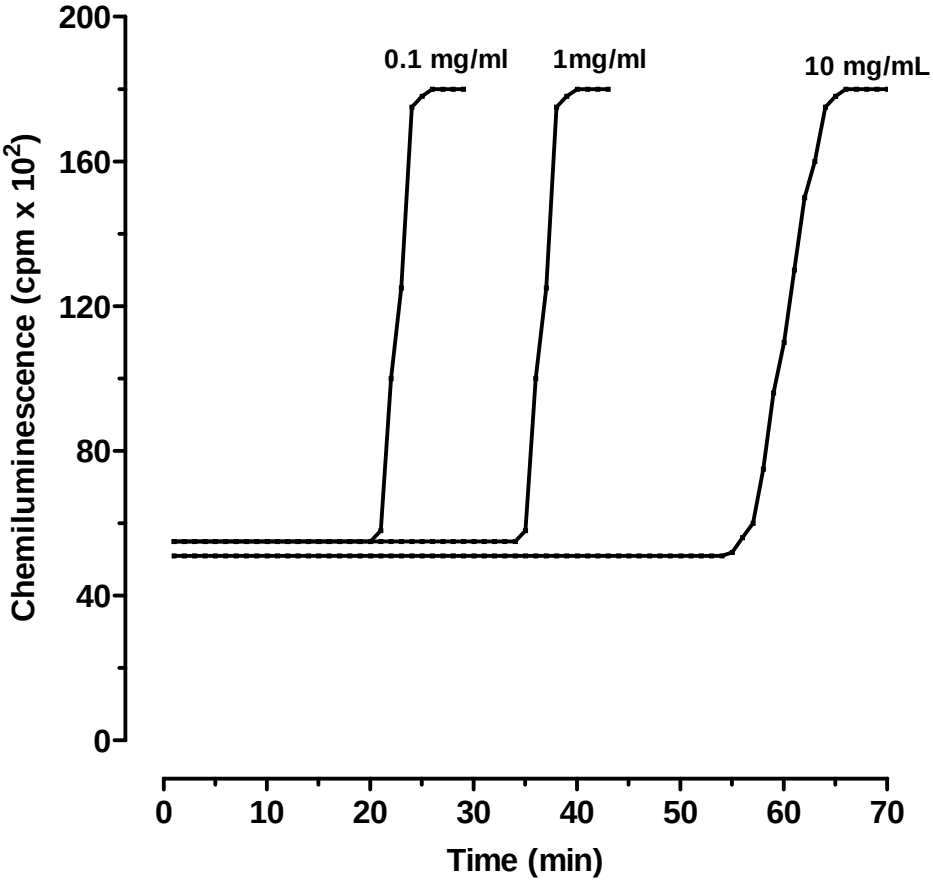

Figure 3. Chemiluminescence intensity measured after addition of 5 µl of different extract concentration of *Eupatorium buniifolium* aqueous extract to tha ABAP luminol system

These differences can be the result of the presence of efficient and inefficient antioxidants in the extract. The concentration of efficient antioxidants can be derived, in Trolox equivalents, from the fast decay extent, while the change in luminol chemiluminiscence measured after a long time can be related to the total amount of antioxidants (efficient and inefficient) present in the sample (19).The measured induction times were proportional to the concentration of the extract, for the most concentrated extract (10 mg/mL) was 463 µM Trolox, 295 µM Trolox for 1/10 dilution and 165 µM Trolox for 1/100 µM Trolox. These equivalent results for TRAP and TAR values may be due to the presence of antioxidants of high reactivity (16) such as phenolic and flavonoid compounds, extensively reported as free radicals scavengers.

Quantitative determination of polyphenols and flavonoids

The total phenolic content was 8.33 ± 2.09 g/100g dried plant material and flavonoid compounds were 4.35 ± 0.38 g/100g dried plant material.

Acute toxicity

The administration of *E. buniifolium* aqueous extract was not lethal even up to an oral dose of 3000 mg/Kg p.o. Therefore, the oral LD_{50} was greater than 3 g/kg in mice.

The extract did not produce any sign of toxicity at the tested doses during the period of observation and at necropsy no macroscopic changes in visceras could be detected in the treated groups. Besides, no significant difference in body weight gain was noted between the control and any of the treated groups at any period time.

Discussion

The results showed that the extract presented significant choleretic effect after both oral and i.v. administration, without changes in bile acids excretion.
Several experimental studies have established that the bile secretion is mainly induced through two mechanisms, defined as bile acid-dependent and independent secretion (20), so the extract enhance bile secretion in rats by increasing the bile acid-independent flow, that is without stimulating bile acid output.
The data reported in the present work indicate that the extract has antioxidant capacity. This activity is more pronunciated in the concentrated extract. This difference can be the result of the presence of several antioxidants of different antioxidant reactivity.
Flavonoids and phenolics of higher plants are known to be excellent antioxidants in vitro and intake of plant polyphenol antioxidants may have positive effects in oxidative-stress related pathologies (21). As several diseases of the gastrointestinal tract seem to be induced by oxidative stress, the antioxidant activity of this extract may have beneficial effects on liver and gallbladder diseases, where reactive oxygen species are involved (22, 23).
This preliminary findings on antioxidant and choleretic activities here reported, lend support to the use of *E. buniifolium* in popular medicine to treat gastrointestinal troubles and as digestive agent.

Acknowledgements

This work was supported in part by UBACYT, Universidad de Buenos Aires.

References

1. Ríos J, Condori L, Muñoz S, Loayza I, Soriano J. The extraction of the essential oil of romerillo. Actes du colloque de Chicoutimi, 1993; 22-25 août,111-117.

2. Rojas Acosta N, Plantas Medicinales de Corrientes. Gadola P, ed. Buenos Aires, 1905:28.

3. Saggese D, Yerbas Medicinales Argentinas: 10th ed, Rosario, 1959:111-115.

4. García G, Campos, R, de Torres R, et al. Antiherpetic Activity of Argentine Medicinal Plants. Fitoterapia 1990;61:542-546.

5. Muschietti L, Gorzalczany S, Ferraro G, Acevedo C, Martino V. Phenolic compounds with anti-inflammatory activity from *Eupatorium buniifolium.* Planta Medica 2001;67:743-744.

6. Miño J., Muschietti, L., Ferraro G, Martino V, Acevedo C. Antinociceptive activity of *Eupatorium buniifolium* aqueous extract. Fitoterapia 2005;76:100-103.

7. Miño J, Muschietti L, Ferraro G, Martino V, Acevedo C. Neuropharmacological activity of *Eupatorium buniifolium* aqueous extract in mice. Phytoterapy Research 2007;21:262-365.

8. Muschietti L, Martino V, Ferraro G, Coussio J. Compuestos polifenólicos aislados de *Eupatorium buniifolium.* An. Asoc. Quim. Argent. 1990;78:329.

9. Muschietti L, Martino V, Ferraro G, Coussio J. 5,7,5'trihydroxy-3,6,2',4' tetramethoxyflavone from *Eupatorium buniifolium.* Phytochemistry 1994;36:1085-1086.

10. Caula S, Villar S, Martino V, Coussio J, Ferraro G. Polyphenols isolated from *Eupatorium buniifolium*. Revista Latinoamericana de Química 1991;22:1-3.

11. Olfert ED, Cross BM. Guide to the care and use of experimental animals In: Canadian Council of Animal Care, 2nd ed., McWilliam, A.A. (Eds) Otawa, Ont CCAC. 1993;1

12. Lee JN, Park CS, Kim HP, Hwang SY, Chung WG. Single dose toxicity of Hwangjaegongjinbo, an invigorator, in mice and rats. Journal of Toxicology and Public Health 2002;18:73-77.

13. Ryu S.D, Park CS, Baek H.M. Anti-diarrheal and spasmolytic activities and acute toxicity study of Soonkijangquebo, and herbal anti-diarrheal formula. Journal of etnopharmacology 2004;94:75-80.

14. Lexa A, Fleurentin J, Lehr P, et al. Choleretic and hepatoprotective properties of *Eupatorium cannabinum* in the rat. Planta Medica 1989;55:127-132.

15. Bruusgaard A, Quantitative determination of the major 3-hydroxy bile acids in biological material after thin-layer chromatographic separation. Clinica Chimica Acta 1970;28:495-504.

16. Lissi E, Salim-Hanna M, Pascual C, del Castillo M. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminiscence measurements. Free Radical Biololy and Medicine 1995;18:153-158.

17. Singleton VL, 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal Enology Vitiviniculture 1965;16:144-158

18. Bonvehi JS, Torrent MS, Lorente EC. Evaluation of polyphenolic and flavonoid compounds in honey pollen producen in Spain. Journal of Agricultural and Food Chemistry 2001;49:1848-1853

19. Campos A, Escobar J, Lissi E. Total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR) of Ilex Paraguayensis extracts and red wine. Journal Brazilian Chemical Society 1996; 7:43-49

20. Okolicsanyi L, Lirussi R, Strazzabosco M. The effect of drugs on bile flow and composition. Drugs 1986;31:430-448

21. Urquiaga I, Leighton F. Plant polyphenol antioxidant and oxidative stress. Biological Research 2000;33:55-64.

22. Blázovics A, Fehér E, Abdel Rahman M, et al. Free radicals in connection of bile and liver. Czech and Slovak Gastroenterology and Hepatology 1996;3:73-78.

23. Oh TY, Lee JS, Ahn BO, et al. Oxidative damages are critical in pathogenesis of reflux esophagitis implication of antioxidants in its treatment. Free Radical Biology and Medicine 2001;30:905-915.