ISOLATION OF ANTIGENOTOXIC URSOLIC ACID FROM *Ixora coccinea* FLOWERS

SEPARACIÓN DEL ÁCIDO URSÓLICO ANTIGENOTÓXICO DE LAS FLORES DE *Ixora coccinea*

PG Latha[1], MNS Nayar[2], OV Singh[2], V George[2], KR Panikkar[1] and P Pushpangadan[1]

Abstract

Ixora coccinea L. (Rubiaceae) is a common shrub distributed throughout tropical India. The flowers of *I. coccinea* are extensively used in ayurvedic medicine. The hexane fraction of the flowers showed significant antigenotoxic properties. Chemical investigation of the above fraction has led to the isolation of the triterpenoid, ursolic acid as the active constituent.

Key words: *Ixora coccinea*, triterpenoid, ursolic acid, antigenotoxic effects.

Resumen

Ixora coccinea L (Rubiaceae) es un arbusto común ampliamente distribuido en la India tropical. Las flores de *I. coccinea* se usan extensamente en la medicina Ayurbédica. La fracción hexano de las flores mostró propiedades antigenotóxicas significativas. El estudio químico de esta fracción ha permitido la separación del triterpenoide, el ácido ursólico como el constituyente activo.

Palabras clave: Ixora coccinea, triterpenoide, ácido ursólico, efectos antigenotóxicos.

INTRODUCTION

The flowers of *Ixora coccinea* are used extensively in ayurvedic medicine to treat dysentery, leucorrhoea, dysmenorrhoea and catarrhal bronchitis (Satyavati *et al.*, 1987). A decoction of the flowers is used against eye troubles, sores and ulcers. Medicated oil prepared from the flowers is used to treat scabies (John, 1984).

Rutin, leucocyanidin glycoside, cyanidin–3–rutinoside, delphinidin monoglycoside and mannitol have been reported from the flowers of *I. coccinea* (Subramanian and Nair, 1971; Rastogi and Mehrotra, 1991).

In continuation of our earlier report on the cytotoxic and antitumour properties of the hexane extract prepared from *I. coccinea* flowers (Latha and Panikkar, 1998), we now report the isolation, characterisation and antigenotoxic effects of the active constituent, ursolic acid on the mouse sperm system.

MATERIALS AND METHODS

I. coccinea flowers were collected from the herbal garden of the Institute. A voucher specimen has been deposited at the Institute's herbarium (TBGT 41601). Shade dried, powdered flowers (100 g) was exhaustively extracted with n-hexane in a soxhlet apparatus for 48 hours. The extract was concentrated and evaporated to dryness under vacuum to obtain a yellow crude extract (4.5 g). The crude extract on column chromatography over Si gel afforded a colourless material (450 mg) on elution with 5% Et OAc in hexane (0.45% yield), which is referred to as IC_1. TLC

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[1] Ethnomedicine and Ethnopharmacology Division, Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram-695562, India. E-mail: telenet@eth.net.

[2] Phytochemistry Division, Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram-695562, India.

was carried out using silica gel G (Sisco Research Laboratories, Bombay). The solvent system used was benzene: ethyl acetate (2:1). Detection of the spot on TLC plate was done using Liebermann–Burchard spray reagent (Wagner *et al.*, 1984). The melting point of IC_1 was determined using a Fischer Johns melting point apparatus. The melting point was reported uncorrected. IR spectrum of IC_1 was recorded on a FTIR NICOLET 510 spectrometer. ^{1}H-NMR of IC_1 in $CDCl_3$ was recorded on a JEOL GSX 400 MH_z spectrometer. The mass spectrum of IC_1 was recorded on a Finnigan MAT 8230 spectrometer.

Antigenotoxic effects

Genotoxic agent used. Potassium dichromate ($K_2Cr_2O_7$, molecular weight 294.21, E. Merck India) was dissolved in water and used as the genotoxic agent.

Test animals. Eight-week-old healthy inbred Swiss albino mice (*Mus musculus*), average body weight 25-30 g, maintained in the animal house of the Institute (original stock obtained from the Sree Chitra Tirunal Institute of Medical Sciences and Technology, Thiruvananthapuram, India) were used for the experiment. The animals were housed in polypropylene cages (290 x 220 x 140 mm) bedded with sterile paddy husk and maintained under standard environmental conditions of temperature (24-28 °C), relative humidity (60-70%) and twelve hours light/dark cycles. Boiled water and commercial mouse pellet diet (Gold Mohur, Lipton, India) were given *ad libitum.*

Experimental protocol. Five groups of animals, 6/group were used for the experiment. IC_1 was made into a uniform suspension in 0.5% gum acacia solution and used for the bioactivity studies. Mice of set 1 were fed 0.5% gum acacia solution (1 ml) and was the control. $K_2Cr_2O_7$ was administered p.o. to set II at a dose of 20 mg/kg body weight. In set III, all the animals were primed with IC_1 (100 mg/kg body weight p.o.) for seven consecutive days before being fed $K_2Cr_2O_7$ solution (20 mg/kg body weight) on day seven, one hour after priming. In set IV, the animals were primed p.o., with IC_1 (100 mg/kg) for seven consecutive days and not given $K_2Cr_2O_7$. Animals were sacrificed by cervical dislocation and dissected out. The reproductive tract was exposed and both the caudae epididymis were removed and placed in a Petri dish containing 2 ml of phosphate–buffered saline (PBS pH 7.2). The resulting suspension was filtered through two layers of cheese cloth to remove tissue debris, stained with 1% aqueous eosin Y (10:1) for 30 min and smears were made on clean dry slides. Sperm count was done using haemocytometor. Slides were also analysed for presence of abnormal sperms according to the criteria of Wyrobek and Bruce (1975). Two thousand sperms from each animal were analysed. Sperms of treated and control animals (three from each group) were examined, 1 week and weeks after clastogen treatment. Testis weight of each animal which forms additional information for spermatogenic damage was found out. Statistical analysis was done using Student's 't' test.

RESULTS AND DISCUSSION

IC_1, m.p. 285 °C, appeared as a single spot on TLC, gave positive Liebermann–Burchard reaction, giving a brick red colour indicating it to be a triterpenoid. IR γ_{max}^{KBr} 3462 (-OH), 1734 (carboxylic), 1385, 1375 (gem dimethyl), 1040 cm^{-1} (C-OH). 400 MHz. ^{1}H-NMR spectrum (in $CDCl_3$) showed seven methyl groups at δ 0.78, 0.79, 0.86, 0.93, 0.95, 0.99 and 1.08, a one proton signal at δ 3.21 (H-3) and an olefenic proton at δ 5.25 (H-12). MS showed the M^+ ion at m/z 456 and other significant fragment ions at m/z 203, 248, 189 and 133. From these data, IC_1 has been identified as ursolic acid (figure 1).

The results obtained for sperm abnormality study are presented in figures 2 and 3. $K_2Cr_2O_7$ administered at a dose of 20 mg/kg body weight induced significantly higher frequency of abnormal sperms compared to the control set. The types of abnormal sperms observed were amorphous, hookless, folded and banana, based on the classification of Wyrobek and Bruce (1975).

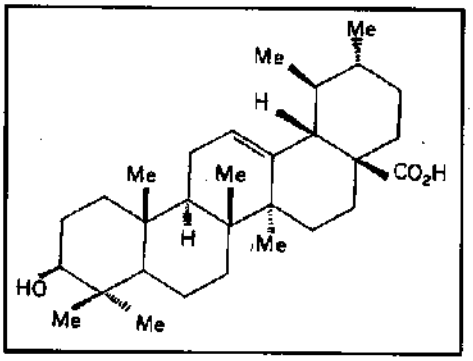

Figure 1. Ursolic acid

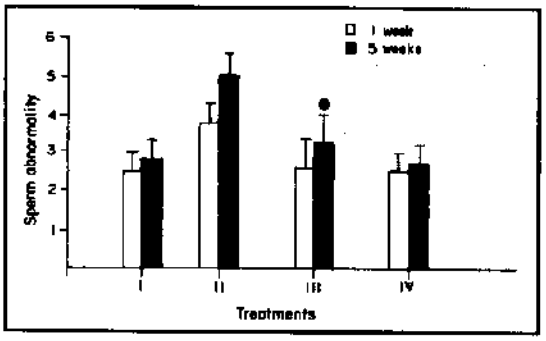

Figure 2. Effect of IC_1 on sperm abnormalities of $K_2Cr_2O_7$ treated mice

Treatment I	0.5% gum acacia
Treatment II	$K_2Cr_2O_7$ (20 mg/kg)
Treatment III	IC_1 (100 mg/kg x seven days) + $K_2Cr_2O_7$ (20 mg/kg)
Treatment IV	IC_1 (100 mg/kg x seven days)

(• P > 0.01)

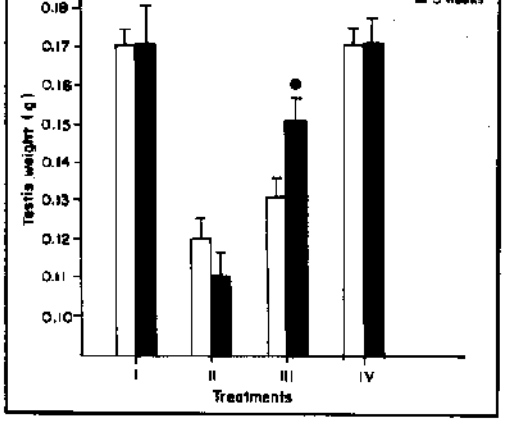

Figure 3. Effect of IC_1 on testis weight of $K_2Cr_2O_7$ treated mice

Treatment I	0.5% gum acacia
Treatment II	$K_2Cr_2O_7$ (20 mg/kg)
Treatment III	IC_1 (100 mg/kg x seven days) + $K_2Cr_2O_7$ (20 mg/kg)
Treatment IV	IC_1 (100 mg/kg x seven days)

(• P > 0.01)

IC_1 when administered alone did not induce sperm abnormalities in significantly higher frequency than the control value at both one week and five weeks. In $K_2Cr_2O_7$ + IC_1 treated group, a significant decrease in percentage of abnormal sperms was observed compared to $K_2Cr_2O_7$ treated group, at both 1 week and 5 weeks. The testis weight of the IC_1 + $K_2Cr_2O_7$ group was higher than $K_2Cr_2O_7$ treated group after five weeks of treatment but it was almost the same in both groups at 1 week (table 1).

The study reveals that IC_1 is capable of protecting the mice from abnormal sperm production caused by $K_2Cr_2O_7$. Even though the mechanism of abnormal sperm production is not known, it is interpreted that the agent which induces abnormalities in sperm is an agent that interferes with either the integrity of the DNA itself or the expression of this genetic material (Wyrobek *et al.*, 1984).

Chromium salts are known oxidising agents causing DNA damage through generation of active oxygen species (Sugden *et al.*, 1990). The interaction of IC_1 and $K_2Cr_2O_7$ leads to protection against the genotoxicity of the latter. Ursolic acid is known to have marked antioxidant properties and is a potent antimutagen (Liu, 1995). In consonance with these findings, magnalol, another plant product has been reported to protect sperm motility by inhibiting lipid peroxidation (Sarker, 1997). IC_1 may protect against the genotoxicity of $K_2Cr_2O_7$ by acting as a scavenger of free radicals or forming inactive complexes, thereby reducing the deleterious effects. The protective effect of crude spinach beet leaf against clastogenicity of $K_2Cr_2O_7$ has already been reported (Sarkar *et al.*, 1996). The present study is particularly relevant because it deals with protection afforded by IC_1 from the genotoxic effects of $K_2Cr_2O_7$ on germinal cells, because this is the only system in which transmissible genetic damage from one generation to another takes place (Au and Hsu, 1980). However, further investigations are necessary to identify its mechanism(s) of action.

Table 1. Effect of IC_1 on testis weight and sperm morphology of $K_2Cr_2O_7$ treated mice

Group	Treatment	Time (weeks)	Testis weight (g)	% sperm with abnormal shape
I	0.5% gum acacia (control)	5	0.16 ± 0.06 [0.16 ± 0.03]	2.70 + 0.1 [2.50 + 0.1]
II	$K_2Cr_2O_7$ (20 mg/kg body weight)	5	0.11 ± 0.07 [0.12 ± 0.03]	5.00 ± 0.7 [3.70 ± 0.2]
III	IC_1 (100 mg/kg body weight) x 7 days + $K_2Cr_2O_7$ (20 mg/kg body weight)	5	0.15 ± 0.06* [0.13 ± 0.03]	3.20 ± 0.6* [2.55 ± 0.3]*
IV	IC_1 (100 mg/kg body weight x seven days)	5	0.16 ± 0.07 [0.16 ± 0.03]	2.60 ± 0.2 [2.54 ± 0.1]

The figures in [ ] are the one week data. * $P < 0.01$

Ursolic acid has previously been reported to possess hepatoprotective, antiinflammatory, antiarthritic, antidiabetic, antiulcer, hypolipidaemic and antiartherosclerotic activities (Liu, 1995). Thus it is a very important phyto-chemical with several therapeutic properties and this is the first report of its antigenotoxic property on the mouse sperm system.

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