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Internal Secretory Structures and Preliminary Phytochemical Investigation on Flavonoid and Coumarin Content in *Santolina insularis (Asteraceae)*

By

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With 9 Figures

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Summary

SACCETTI G., ROMAGNOLI C., BALLERO M., TOSI B. & POLI F. 1997. Internal secretory structures and preliminary phytochemical investigation on flavonoid and coumarin content in *Santolina insularis (Asteraceae).* – Phyton (Horn, Austria) 37 (2): 219–228, 8 figures. – English with German summary.

UV light investigation has shown that vegetative and flowering plants of *Santolina insularis* (FIORI) ARRIG. have internal secretory structures similar to those of many other *Asteraceae.* In the root two arrays of ducts arise abutting the endodermis, while in the stem single or unconnected pairs of ducts are localized between the vascular bundles. The leaves and bracts present ducts vein-associated. A preliminary phytochemical investigation employing HPTLC evidenced the presence of some fluorescent flavonoids and coumarins. The fluorescence of the secretion and the presence of fluorescent metabolites in the plant organs, lead to assume that the secretory system is involved in producing and/or storing the secondary metabolites which justify the use of this species in Sardinian traditional medicine.

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Zusammenfassung

SACCHETTI G., ROMAGNOLI C., BALLERO M., TOSI B. & POLI F. 1997. Innere sekretorische Strukturen und vorläufige Untersuchungen über den Flavonoid- und Cumarin-Gehalt von *Santolina insularis (Asteraceae)*. – Phyton (Horn, Austria) 37 (2): 219–228, 8 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Untersuchungen mit UV-Licht haben ergeben, daß sowohl vegetative als auch blühende Pflanzen von *Santolina insularis* (FIORI) ARRIG. innere sekretorische Strukturen besitzen, welche ähnlich denen vieler anderer *Asteraceaen* sind. In der Wurzel sind zwei Reihen von Kanälen vorhanden, welche an die Endodermis angrenzen, während im Stamm einzelne oder unzusammenhängende Paare von Kanälen zwischen den Gefäßbündeln angeordnet sind. Bei den Blättern und den Deckblättern treten die Gänge gemeinsam mit den Adern auf. Eine vorläufige phytochemische Studie mittels HPTLC Methode zeigte, daß einige fluoreszierende Flavonoide und Cumarine vorhanden sind. Die Fluoreszenz des Sekrets und die Anwesenheit von fluoreszierenden Stoffwechselprodukten in den Pflanzenorganen führen zur Annahme, daß das sekretorische System in die Produktion und/oder Speicherung von sekundären Metaboliten eingebunden ist. Das Vorhandensein derartiger sekundärer Stoffwechselprodukte rechtfertigt die Verwendung dieser Art in der traditionellen Heilkunde Sardiniens.

Introduction

Asteraceae is a family of flowering plants with a complex glandular system supplying many bioactive compounds (LERSTEN & CURTIS 1987). Studies regarding the genus *Santolina* report that the aerial parts of the plants have numerous usable secondary metabolites such as flavonoids (BECCHI & CARRIER 1980), essential oils (SENATORE & DE FEO 1994), coumarins (MAQUA & al. 1988) and polyacetilenic compounds (UTRILLA & al. 1995, CHRISTENSEN 1992).

Different species of *Santolina* are employed in folk medicine in the Mediterranean area (DUKE 1987, GRIEVE 1980). One of these species is *Santolina insularis* (GENN. ex FIORI) ARRIG., a plant endemic to the island of Sardinia distributed in an area including the massifs of Gennargentu (Central Sardinia) and Marganai-Linas (South-East Sardinia). The secretory tissues of this plant were studied because Sardinian folk medicine reports many therapeutic uses for the aerial parts of mature plants in full bloom including vermicide, insecticide and remedies against poisoning (BALLERO & FRESU 1991, BALLERO & al. 1995). To ascertain the validity of these applications the presence and distribution of the internal glandular system was studied. In addition, a phytochemical investigation was performed using HPTLC (High Performance Thin Layer Chromatography) to evaluate the presence of bioactive coumarins and flavonoids.

Materials and Methods

Plant material

Mature plants of *Santolina insularis* (GENN. ex FIORI) ARRIG. were collected during the vegetative phase, before flowering (March) and while in full bloom (June), on the Marganai mountains in the district of Cagliari (700 m above sea level, 39° 21′ Latitude North, 8° 34′ Longitude East). Samples were taken from 10 plants before flowering and from 10 plants in full bloom and processed for the examination of coumarin and flavonoid contents and for histological analysis.

Conventional and fluorescent microscopy

Small pieces of roots, stems, leaves and flower heads were fixed and embedded following the procedures described in POLI & al. 1995a. Fresh sections were mounted in water and viewed directly using a fluorescence microscope. For all the conventional and fluorescence observations a Zeiss Axiophot Photomicroscope equipped with UV exciting illumination (UV-H 365 : BP 365/12, FT 395, LP 397) was employed.

Phytochemical investigation

15 g of each sample of roots, stems, leaves and flower heads were placed in 300 ml ethanol for 2 days. Successively, they were filtered and evaporated to 10 ml under reduced pressure at 40–45 °C. Then, the samples were chromatographed. NP (Normal Phase) plates for HPTLC (Merk 5641), silica gel 60, 10 × 20 cm, were used. The plates were developed in a Camag chromatographic chamber in two steps employing two different solvent solutions. First step (running solvent solution 1) = ethyl acetate: formic acid : acetic acid:water (100 : 11 : 11 : 27). Second step (running solvent solution 2) = toluene : ethyl acetate : acetic acid (50 : 45 : 5). Both commercial standards and sample solutions were applied to the plates as 10 mm wide bands employing a Linomat IV, automatic TLC sampler (10 µl, delivery speed 4 sec/µl). Coumarins gave an inherent bluish fluorescence. To detect flavonoids, the plates were sprayed with a watery solution of alluminium sulphate 4 %. Flavonoids exhibited different nuances in colour from yellow to yellow-green. The plates were scanned with a Camag Scanner II equipped with CATS software 3, Camag. Fluorescence was induced at 366 and 340 nm with a mercury vapour lamp. Emission was measured through a cut-off filter (400 nm), sensitivity 210–220, span 7, offset 5 %. Flavonoids and coumarins were identified by chromatographic comparison of the bands and by correlation between the UV-spectra of the samples and that of commercial standards. The flavonoid and coumarin standards employed were: hesperidin (Sigma Chemical Company, St. Louis, USA), luteolin-7-glucoside, apigenin-7-glucoside, apigenin, xanthotoxin (Roth, Karlsruhe, Germany), scopoletin (Aldrich Chemie, Steinheim, Germany). As for HPTLC quantitative analysis, calibration graphs were obtained with 5 µl of various dilute standards, with at least seven applications of each dilution. Quantification of the compounds was performed by peak integration. Each value was the average of 10 determinations ± standard deviation (s.d.). The regression lines were calculated by the method of least squares. All calibration curves had a correlation coefficient of at least 0.98.

Results

Secretory reservoirs were observed in all the plant organs both before flowering and while in full bloom. The structures showed a yellow-brown secretion under conventional microscopy and a yellow-blue fluorescence under UV light (365 nm). The roots of *Santolina insularis* plants gathered before flowering revealed two arrays of secretory structures (Fig. 1) holding a secretion which emitted a bright yellow-blue fluorescence (Fig. 2).

Seriate freehand and resin cross sections made it possible to observe that the ducts, always present along the full length of the root, were unbranched and unconnected. In the stem, the varying number of reservoirs was always located near the vascular bundles below the cortical area (Fig. 3). Under UV excitation, the ducts exhibited an evident secretory epithelium formed by small cells with a secretion fluorescing bright yellow-blue (Fig. 4). As in the roots, the canals were unbranched and unconnected (Fig. 5). The secretory structures were absent in the petiole, but near the main leaf vein there were two reservoirs (Fig. 6). Under UV microscopy, a bright yellow fluorescence, mainly of the secretory product, was visible, while the epithelium fluoresced weakly (Fig. 7).

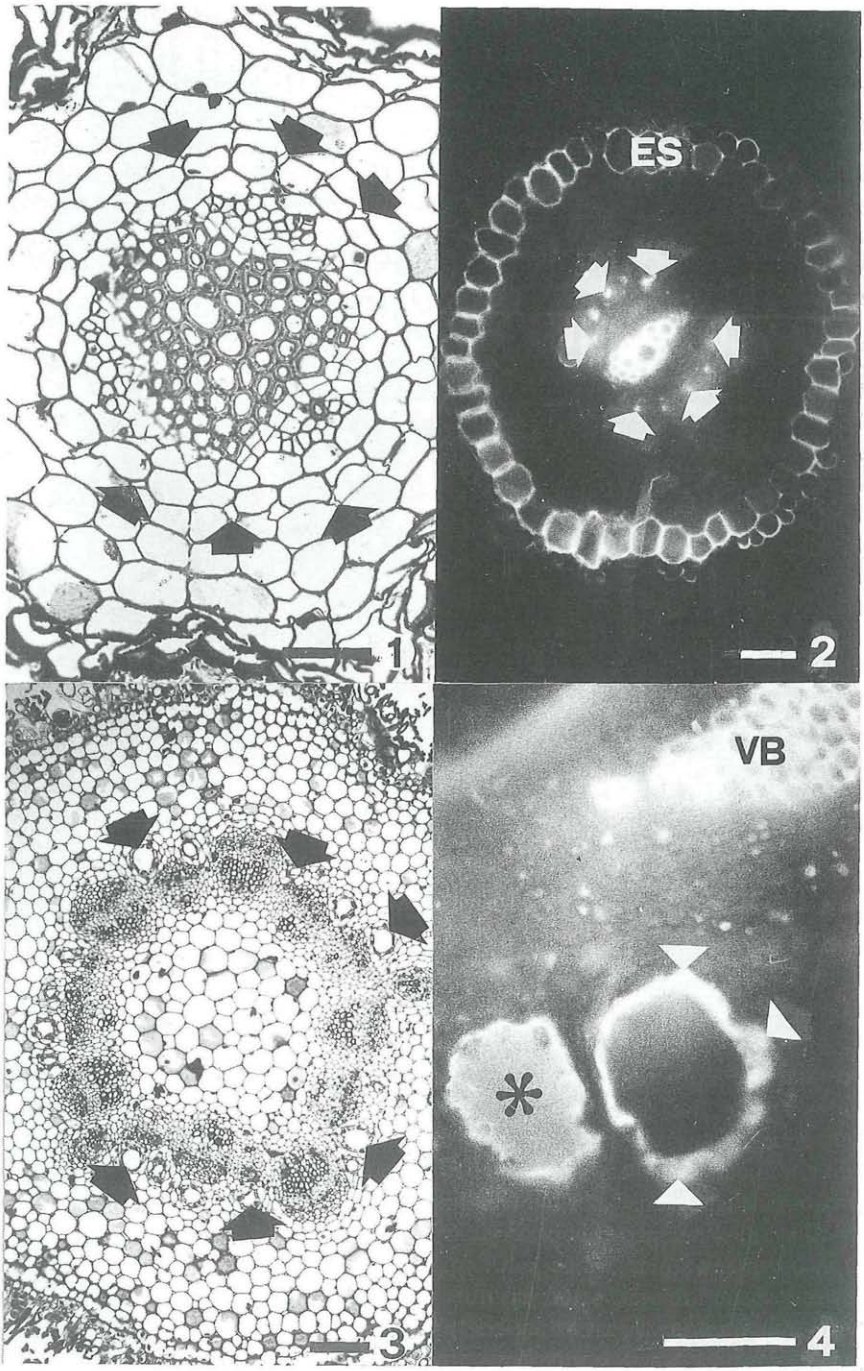
In full flowering plants, the structure of the secretory system was the same as the one already described for plants in the vegetative phase. In flower heads, the tubular flowers were individually examined and no secretory structures were recognized in either the corolla or the sexual apparatus (androecium and gynoecium). However, in flower bracts secretional reservoirs were present. Seriate transections of fresh and resin bract samples displayed one unbranched duct per bract, located along the full length of the organ and typically placed adjacent to the vascular bundles. Even in this case, the UV observations revealed a bright yellow-blue fluorescence of the secretion content and a weak fluorescence of the secretory epithelium (Fig. 8).

Fig. 1. Resin cross section of a thin root stained with toluidine blue O (TBO). Note the two arrays secretory structures just near the endodermis (arrows = secretory space). Scale bar = 50 μm.

Fig. 2. Freehand cross section under fluorescence microscopy shows two arrays of five secretory structures with a yellow-blue autofluorescence of the secretion (arrows). (ES = esoderm). Scale bar = 50 μm.

Fig. 3. Resin cross section of the stem showing numerous secretory structures (arrows) localized near vascular bundles. Scale bar = 100 μm.

Fig. 4. Freehand cross section of the stem under fluorescence microscopy. Under UV light the fluorescence of the secretion (asterisk) and of the secretory epithelium is evident (arrowheads) (VB = vascular bundles). Scale bar = 50 μm.



As for the preliminary phytochemical investigation, employing HPTLC, it was found that some flavonoids and coumarins were present in all the organs of *S. insularis* plants, both in the vegetative stage and in full bloom (Table 1). The comparison between the UV-spectra of the chemical standards with that of the samples led to identify the flavonoids herniarin, luteolin-7–glucoside, apigenin, apigenin-7-glucoside and the coumarins scopoletin and xanthotoxin (Fig. 9). In the pre-flowering plants, hesperidin was the flavonoid having the highest concentration, while scopoletin was the most abundant coumarin detected. In full flowering plants, the highest flavonoid concentrations were found in the flowers, about 2–4 fold higher than in the stems and leaves. As for scopoletin, its concentration was higher in all the organs of the flowering plants as compared to those in the vegetative stage. For of all the chemicals analyzed the highest concentrations were registered in the aerial parts of the plants.

Discussion

Under both fluorescence and conventional microscopy, the complex system of secretory structures in *Santolina insularis* shows internal reservoirs (ducts) with secretory epithelium and secretion emitting a typical bright yellow-blue fluorescence at 365 nm. This particular kind of emission has previously been observed in other plants in which the presence of fluorescent coumarins and flavonoids were ascertained (POLI & al. 1995b).

The morphology and distribution pattern found in *S. insularis* present many similarities with the secretory systems outlined in other *Asteraceae*. Specifically, the same unbranched and unconnected structures have been described in both *Ambrosia trifida* and *Tagetes patula*, particularly for the roots and stems (LERSTEN & CURTIS 1988, POLI & al. 1995a).

The phytochemical investigations and fluorescence microscopy indicate that the bright yellow-blue fluorescence in the ducts of fresh sections is most likely due to the appreciable amounts of coumarins and flavonoids

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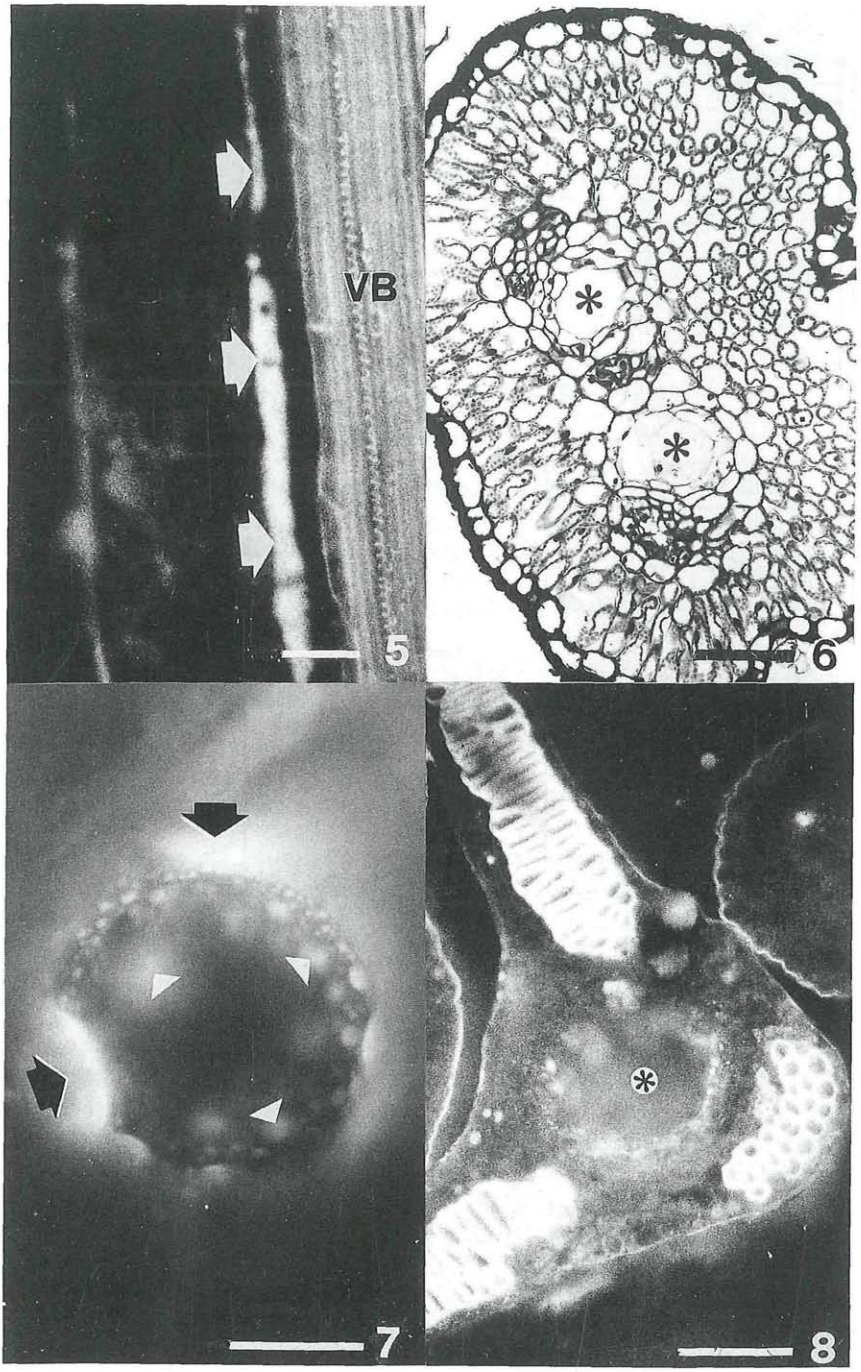
Fig. 5. Longitudinal freehand section of the stem at fluorescence microscope. Note the fluorescence of the secretory structures (arrows) (VB = vascular bundles). Scale bar = 100 μm.

Fig. 6. Cross section of a leaflet in a resin sample stained with toluidine blue O (TBO) showing two reservoirs (asterisks) running close to the minor vein. Scale bar = 100 μm.

Fig. 7. Freehand cross section under UV light of a leaflet. Note the bright fluorescence of the secretory product inside the cavity (arrowheads) and partially of the secretory epithelium (arrows). Scale bar = 50 μm.

Fig. 8. Freehand cross section of the bract under UV microscopy showing a reservoir (*) with a fluorescent secretion adjacent to vascular bundles. Scale bar = 100 μm.



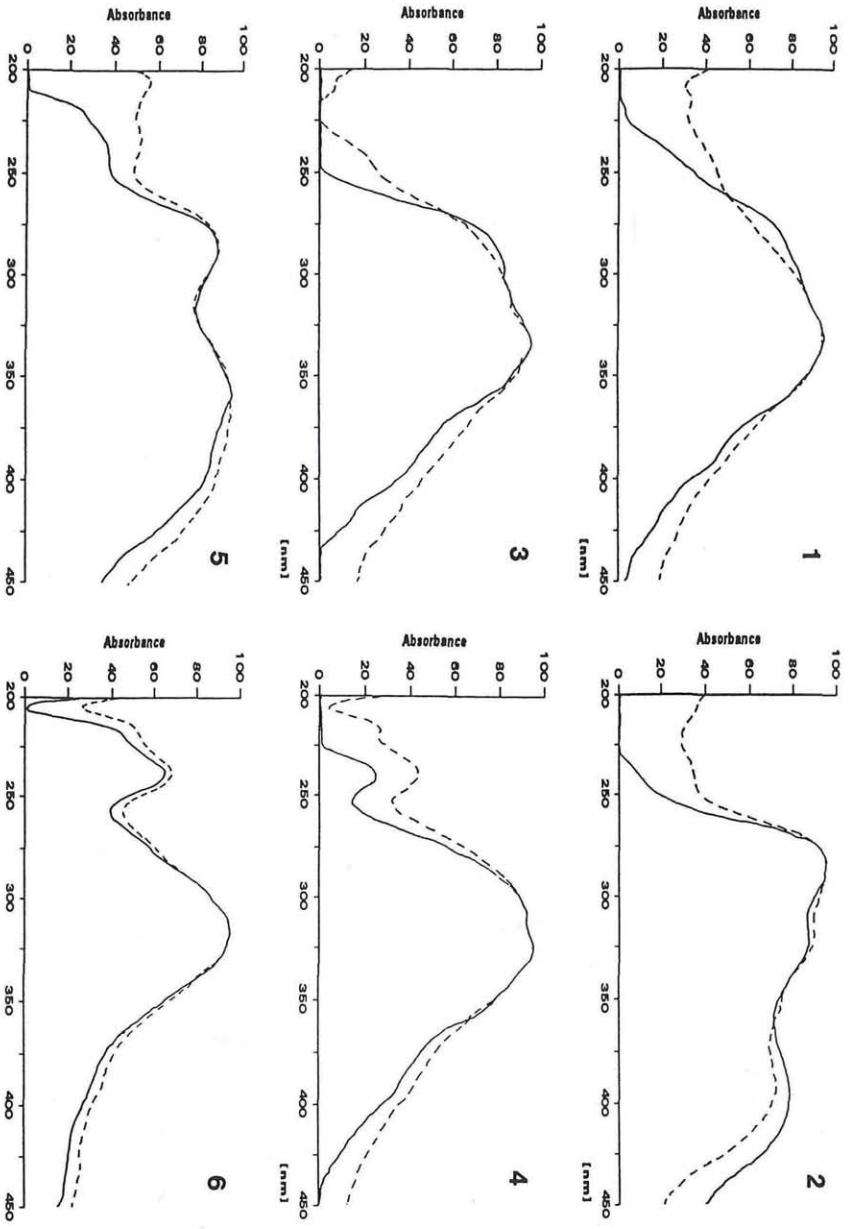


Fig. 9. UV-spectra in reflectance measurement of flavonoid and coumarin commercial standards (full line) and of the samples (dotted line). The coincidence of the curves led to identify the flavonoid and coumarin compounds in the samples: **1**. hesperidin; **2**. luteolin-7-glucoside; **3**. apigenin-7-glucoside; **4**. agigenin; **5**. scopoletin; **6**. xanthotoxin.

Table 1.

Presence of coumarins and flavonoids in the organs of *Santolina insularis* plants collected before flowering and during full blossom.

S. insularis (March)			
flavonoids	roots	stems	leaves
hesp	8.9±1.35	11.8±1.68	12.23±1.87
l-7-g	1.8±0.39	1.3±0.28	3.3±0.59
a-7-g	3.1±0.68	2.7±0.56	3.4±0.61
ap	0.23±0.021	0.33±0.028	0.54±0.033
coumarins			
sco	0.087±0.005	0.198±0.056	0.326±0.027
xth	0.015±0.002	0.011±0.001	0.053±0.004

S. insularis (May)				
flavonoids	roots	stems	leaves	flowers
hesp	6.35±0,93	12.73±1.89	16.48±2.09	57.15±4.42
l-7-g	0.6±0.060	1.0±0.22	1.74±0.34	7.89±1.1
a-7-g	1.88±0.39	1.74±0.35	3.15±0.57	12.9±1.91
ap	0.104±0.015	0.162±0.018	0.3±0.029	0.76±0.067
coumarins				
sco	0.217±0.030	0.362±0.039	0.409±0.051	1.250±0.32
xth	0.02±0.0015	0.02±0.0015	0.038±0.0031	0.076±0.0012

hesp: hesperidin; l-7-g: luteolin-7-glucoside; a-7-g: apigenin-7-glucoside; ap: apigenin; sco: scopoletin; xth: xanthotoxin. All the determinations are expressed in mg/g fresh weight ± standard deviation

present in these structures. In fact, under the same excitation conditions (365 nm), standard samples of xanthotoxin, hesperidin, luteolin-7-glucoside, apigenin-7-glucoside and apigenin all emit the same fluorescence (Poli & al. 1995b). On the other hand, the weak blue fluorescence detected in the secretory ducts could be due to scopoletin, although one cannot rule out the presence of polyacetylenic compounds (i.e. thiophenes) which have been ascertained in some other Mediterranean *Santolina* species (Utrilla & al. 1995, Christensen 1992). Likewise, the yellow fluorescence at 365 nm and the yellow-brown secretion under conventional light could be also related to the presence of essential oils. This hypothesis is supported by a detailed phytochemical investigation of the presence and chemical composition of the essential oil in *Santolina insularis* (Poli & al. 1997).

In *Asteraceae* family there is no standard pattern for the secretory systems storing and/or producing different classes of compounds. There are species such as *Ambrosia trifida* with two different secretory structures for the two classes of secretion (Lersten & Curtis 1988) and other species, such as *Tagetes patula*, with a single structure producing and storing both es-

sential oils and polyacetylenic compounds (Poli & al. 1995a). The present UV detection data suggest that in *S. insularis* the secretory system is involved in the accumulation and/or synthesis of various kinds of compounds.

In the aim of better defining the morpho-functional pattern of *S. insularis* secretory structures, more detailed phytochemical analyses are in progress.

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