# 1,4-Benzoxazin-3-one, 2-Benzoxazolinone and Gallic Acid from *Calceolaria thyrsiflora* Graham and their Antibacterial Activity

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Secondary metabolites, DIBOA, HBOA, 7-OH-HBOA, BOA and gallic acid, were isolated and quantified from *Calceolaria thyrsiflora* Graham, a native medicinal plant of Chile belonging to the Scrophulariaceae family. The highest DIBOA contents were determined in leaves (145 mmol $kg^{-1}$ dry wt) and flowers (161 mmol $kg^{-1}$ dry wt). Antibacterial activities of DIBOA, HBOA, BOA, gallic acid and infusions of flowers and leaves were determined. The phytomedicinal properties attributed to *C. thyrsiflora* Graham could be understood on the basis of its antibacterial activity.

*Key words: Calceolaria thyrsiflora* Graham, Hydroxamic Acid, Antibacterial Activity

## Introduction

A group of secondary plant metabolites, 1,4-benzoxazin-3-ones (Fig. 1), is mainly known from various wild and cultivated Gramineae (Niemeyer, 1988; Sicker and Schulz, 2002). These compounds, present as glucoside in the tissues, have been implicated as a natural defense factor against insects, fungi and bacteria in the plants. They also have a broad pharmacological profile, which includes antifungal, antibacterial, anticancer and anti-inflammatory activities (Bravo and Lazo, 1993; Roberts *et al*., 1998; Otzuka *et al*., 1988). Cell damage leads to enzymatic hydrolysis of the glucosides yielding free aglycones (Sicker *et al*., 2000; Sicker and Schulz, 2002). The aglycones are considered to be responsible for the biological properties. These molecules are unstable and decompose in solution to the respective 2-benzoxazolinones (see Fig. 1) (Smissman *et al*., 1972; Woodward *et al*., 1978; Bravo and Niemeyer, 1985).

Several of these compounds have also been isolated from some medicinal plants (Pratt *et al*., 1995; Kanchganapoom *et al*., 2001; Ozden *et al*., 1992; Alipieva *et al*., 2003; Bravo *et al*., 2003) and as such they are of significant scientific interest. Finding high concentrations depends on the variety, organ, environmental effects and developmental stage of the plant. All these reasons should be considered in the study of the possible pharmacological role of the 1,4-benzoxazin-3-ones from medicinal plants.

*Scoparia dulcis* L. is a species of the Scrophulariaceae family known as a folk-medicine which has been used in some countries for the treatment of hypertension, diabetes, stomach diseases, and to protect the gastrointestinal system. From this plant DIMBOA, DIBOA, MBOA and BOA (Fig. 1) have been identified (Pratt *et al*., 1995) as well as tetracyclic diterpenes (Chen and Chen, 1976; Hayashi *et al*., 1987, 1991). Biological activity has been related to the diterpenoid structures (Hayashi *et al*., 1991; Betancur-Galvis *et al*., 2001).

*Calceolaria thyrsiflora* Graham is a native Chilean folk-medicinal species (Scrophulariaceae) that has also yielded diterpenoids (Chamy *et al*., 1991) and 3,4,5-trihydroxy benzoic acid (gallic acid) (Selman, 1939). Infusions of flowers and leaves are used in the treatment of hemorrage, diabetes, herpes, throat deseases, shap and as a sweetener. Some of these biological properties are related to the gallic acid contents. In this work we report the contents of DIBOA, BOA, HBOA, 7-OH-HBOA and gallic acid in flowers and leaves of *C. thyrsiflora* Graham. In addition, the antibacterial activities of the pure metabolites and infusions of leaves and flowers are reported

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## Experimental

### *Plant material*

*Calceolaria thyrsiflora* Graham was collected from VII Region, Chile (35° 04' S; 71° 09' W) in October 2003. A voucher sample is on deposit at the Talca herbarium under N° 2736 (cod. Ajim).

### *Chemicals*

The DIBOA standard was isolated from extracts of rye shoots (*Secale cereale* cv. Tetra-Baer) as previously described (Queirolo *et al*., 1983; Lyons *et al*., 1988). 7-OH-HBOA was isolated from extracts of *Stenandrium dulce* (Nees) (Bravo *et al*., 2004). The BOA and gallic acid standards were commercially available products (Aldrich Chemical Co). The HBOA standard was synthesized as previously described (Matlin *et al*., 1979).

### *Chemical analysis*

Contents of DIBOA, BOA, HBOA, 7-OH-HBOA and gallic acid from dry flowers and leaves of *C. thyrsiflora* Graham were quantified by a RP-HPLC method previously described (Bravo *et al*., 2004).

### *Isolation and thin-layer chromatography*

Leaves and flowers (10 g dry wt) of *C. thyrsiflora* Graham were macerated in water (200 ml) at room temperature for 48 h. Extracts were acidified to pH 3.0 with 0.1 N $H_3PO_4$. The solutions were extracted once with hexane and three times with ethyl acetate (300 ml each time). Ethylacetate/petroleum ether (4:1 v/v) was used for the thin-layer chromatography (TLC) on silica gel. The spots were visualized under short-wave length UV light. Ferric chloride developing was used for the preliminary identification of hydroxamic acid and gallic acid.

The compounds were isolated using preparative TLC from ethyl acetate extracts. $R_f$ values of DIBOA, BOA, HBOA, 7-OH-HBOA and gallic acid (Table I) were identical to those of the authentic samples. Melting points and spectroscopic data were in agreement with those reported (Woodward *et al*., 1979; Ozden *et al*., 1992; Glawischning *et al*., 1997; Bravo *et al*., 2004).

### *Infusions*

Infusions *of C. thyrsiflora* Graham were prepared from 5 g of dry flowers or leaves by suspension in 100 ml of water, left for 24 h at room temperature, and then heated for 15 min at 70–80 °C.

### *Antibacterial test*

*Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were grown in Müller-Hinton nutrient medium (DIFCO) and *Streptococcus mutans* (Faculty of Odontology, University of Chile) was grown in Tood Hewitt Broth nutrient medium (DIFCO).

Infusions and pure DIBOA, BOA, HBOA and gallic acid were dissolved in nutrient media and *in vitro* serial dilutions were incubated at 35 °C for 24 h in test tubes containing $10^4$ colony-forming units (CFU). The growth of the microorganisms was examined as a function of turbidity, measured spectrophotometrically at 600 nm.

Percentage inhibition was obtained from: $\% I = 100 (T_s - T_c)/(100 - T_c)$, where $T_s$ is the sample transmittance and $T_c$ the control transmittance.

## Results and Discussion

### *Contents of DIBOA, BOA, HBOA, 7-OH-HBOA and gallic acid*

DIBOA, BOA, HBOA, 7-OH-HBOA and gallic acid (Fig. 1) were subjected to preliminary assessments by TLC using authentic standards. All of them were easily detected as dark zones under 254 nm UV light. DIBOA and gallic acid were visualized with ferric chloride reagent which gives violet and dark-bluish colours, respectively. The $R_f$ values are shown in Table I. The compounds isolated by preparative TLC and the melting points and spectroscopic data were consistent with those reported (Woodward *et al*., 1979; Ozden *et al*., 1992; Glawischning *et al*., 1997; Bravo *et al*., 2004).

Table I. TLC $R_f$ values and HPLC retention time ($R_t$) of DIBOA, HBOA, 7-OH-HBOA, BOA and gallic acid from *C. thyrsiflora* Graham.

| Compound | $R_f$ | $R_t$ [min] |
|---|---|---|
| DIBOA | 0.46 | 8.30 ± 0.32 |
| HBOA | 0.55 | 7.71 ± 0.18 |
| 7-OH-HBOA | 0.42 | 5.50 ± 0.21 |
| BOA | 0.73 | 14.50 ± 0.91 |
| Gallic acid | 0.32 | 3.13 ± 0.05 |

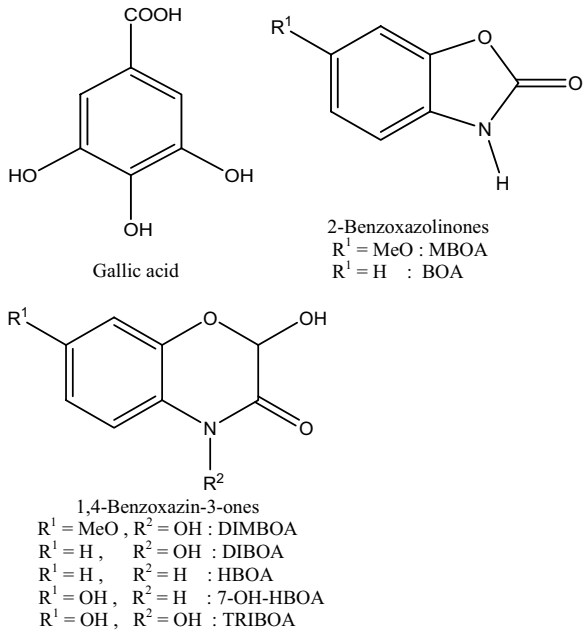


Fig. 1. Structures of 1,4-benzoxazin-3-ones, 2-benzoxazolinones and gallic acid.

An analytical HPLC method, described previously (Bravo *et al*., 2004), was used for the quantitative determination of the compounds. Retention times ($R_t$) of the standards (Table I) were in agreement with those seen for the natural mixture. Contents of DIBOA, BOA, HBOA, 7-OH-HBOA and gallic acid in flowers and leaves of *C. thyrsiflora* Graham are included in Table II.

The contents in the flowers were higher than in the leaves, with the exception of BOA, that was not detected in the flowers. DIBOA was the main aglycone in both organs. DIMBOA and MBOA were not detected. This is the first time that the aglycones HBOA and 7-OH-HBOA have been isolated together from extracts of Scrophulariaceae species. The contents of 7-OH-HBOA were lower than those found for HBOA in flowers and leaves, but in both cases, the content in flowers was always higher than in leaves. Previously, they were also quantified together from an Acanthaceae species (Bravo *et al*., 2004). This could be related to an analogous biosynthetic pathway of DIBOA and DIMBOA in the two species.

Table II. Contents of DIBOA, HBOA, 7-OH-HBOA, BOA and gallic acid in *C. thyrsiflora* Graham.

| Compound | Leaves [mmol/kg dry wt] | Flowers [mmol/kg dry wt] |
|---|---|---|
| DIBOA | 145.4 ± 17.0 | 161.4 ± 7.6 |
| HBOA | 16.0 ± 3.4 | 52.2 ± 6.7 |
| 7-OH-HBOA | 4.4 ± 0.6 | 14.6 ± 1.2 |
| BOA | 56.6 ± 6.9 | nd |
| Gallic acid | 7.1 ± 1.4 | 9.7 ± 2.5 |

nd, not detected.

A proposed biosynthetic route of these compounds (Glawischning *et al*., 1997; Desai *et al*., 1996; Frey *et al*., 2003) suggests that HBOA should be the last precursor of DIBOA. The high contents of HBOA suggest that the enzyme producing the *N*-hydroxylation may be less active in *C. thyrsiflora* Graham.

On the other hand, DIMBOA could be arise from methylation of the putative TRIBOA precursor (Fig. 1). This intermediate may be produced from DIBOA (Frey *et al.*, 2003) or 7-OH-HBOA (Bravo *et al*., 2004). Both routes should be not expressed in *C. thyrsiflora* Graham; for this reason DIMBOA could be not present in this species. But, more experiments will be necessary to clarify fully these possibilities.

*Antibacterial activity*

Table III lists the antibacterial activity of DIBOA, HBOA, BOA, gallic acid and infusions of leaves and flowers from *C. thyrsiflora* Graham. DIBOA displays bacterial inhibitions reaching 100% for *S. aureus*, 49.2% for *E. coli* and 77% for *S. mutans* at the highest concentration tested (1000 $\mu$g/ml).

Table III. Percentage of microbial inhibition (%*I*) by pure DIBOA, BOA, HBOA, gallic acid and *C. thyrsiflora* Graham infusions.

| Substrate | Concentration [$\mu$g ml$^{-1}$] | %*I* *S. aureus* | *E. coli* | *S. mutans* |
|---|---|---|---|---|
| DIBOA | 1000 | 100 | 49.2 | 77.0 |
| | 500 | 27.0 | 0.0 | 36.0 |
| | 250 | 0.0 | 0.0 | 16.0 |
| HBOA | 1000 | 43.7 | 15.3 | 16.0 |
| | 500 | 0.0 | 0.0 | 11.0 |
| | 250 | 0.0 | 0.0 | 0.0 |
| BOA | 1000 | 39.4 | 37.5 | 69.5 |
| | 500 | 46.3 | 0.0 | 27.8 |
| | 250 | 0.0 | 0.0 | 14.1 |
| Gallic acid | 1000 | 100 | 8.6 | 0.0 |
| | 500 | 69.0 | 0.0 | 0.0 |
| | 250 | 67.2 | 0.0 | 0.0 |
| Flowers | 5 g/100 ml | 100 | 0.0 | 100 |
| Leaves | 5 g/100 ml | 65.0 | 0.0 | 60.0 |

In the lower dose range (250 $\mu$g/ml) the percentage of inhibition decrease to 0% for *S. aureus* and *E. coli* and 16% for *S. mutans*.

Only gallic acid showed significant activity against *S. aureus* (67% of inhibition) at lower dose. An opposite result was obtained when the antibacterial activity against *S. aureus* and *E. coli* was performed by dispensing into petri dry surface of the agar diskes (Feijo de Souza *et al*., 2004).

Infusions of flowers display 100% inhibition against *S. aureus* and *S. mutans* and the activity of infusions of leaves was lower in both bacteria.

DIBOA, BOA and HBOA showed significant activity against *S. mutans*. The rank order of potencies DIBOA > BOA > HBOA was observed at the different concentrations. *S. mutans* is the most commonly cariogenic bacterium in humans. Therefore, these results represent a new interesting phytochemical subject of the medicinal properties of *C. thyrsiflora* Graham.

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