FLABELLIFERINS, STEROIDAL SAPONINS FROM PALMYRAH (BORASSUS FLABELLIFER L.) FRUIT PULP

11. Preliminary investigations of effect on yeast and selected bacteria

J.K. NIKAWALA, S.C. WIJEYARATNE, E.R. JANSZ* and A.M. ABEYSEKERA
University of Sri Jayewardenepura, Gangodawila, Nugegoda.

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Abstract: The isolation and partial characterization of four naturally occurring steroidal saponins (flabelliferins) differing in their carbohydrate moiety and isolated from palmyrah fruit pulp was reported previously. These were called flabelliferin F-II (a tetraglycoside), flabelliferin F₆ and F₉ (triglycosides) and flabelliferin F₁₁ (a diglycoside).

On testing for bioactivity, F-II, the bitter saponin (250 µg ml⁻¹) inhibited yeast (Saccharomyces cerevisiae S11- F₆) growth to the extent of 50 - 75% while F₆ (60 µg ml⁻¹) inhibited growth completely. F₆ and F₁₁ were inactive. F₆ and F-II slowed the rate of alcoholic fermentation (F₆ being most potent). But F₉ and F₁₁ did not have any effect on alcoholic fermentation. However none of the 4 saponins affected the efficiency of alcoholic fermentation (86 - 90%).

Antimicrobial studies of flabelliferins using the Bauer - Kirby method showed that only F₆ was active inhibiting all bacteria tested, namely; Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa, Proteus rettigeri and Acinetobacter calcoaceticus. The study is of interest in the area of structure activity relationships, as F₆ (active) and F₉ (inactive) are isomers (M.W. 868).

Key words: Antimicrobial activity, flabelliferins, palmyrah, Saccharomyces cerevisiae, saponins.

INTRODUCTION

Very little work has been done on palmyrah fruit pulp (PFP) although it is available in abundance.¹ Its free use as a food is detracted by the presence of bitterness.¹ A steroid from palmyrah and its monoglucoside and monorhamnoside were isolated from cultivars from Jaffna.² In that study no reference was made to bitterness. A cultivar from Kalpitiya showed the presence of two steroidal glycosides (called flabelliferins) which were labelled F-I and F-II.³ F-I was a tetraglucoside (M.W. 1080) and F-II was a tetraglycoside (M.W. 1030) with two rhamnos and two glucose in its carbohydrate moiety.³,⁴ The bitterness could be removed by the enzyme naringinase ³,⁴ (or heat stable α-amylase in specimens of PFP from Hambantota)⁴ to give spots at higher Rf values. PFP from Hambantota also showed the presence of 3 other flabelliferins called F₁₁, F₆, & F₉ which were isolated by flash chromatography.⁴

* Corresponding author
Flabelliferins \( F_n \) & \( F_c \) were triglycosides (M.W.868) and flabelliferin \( F_a \) a diglycoside (M.W.722). \( F_n \) was found to be most foam stabilizing and haemolytic. Investigations had also shown that boiled PFP did not ferment well but the ability to ferment was restored by incubating with the debittering enzyme naringinase.

Further as PFP does not spoil easily it was speculated that the flabelliferins were bioactive. In the present study the action of the flabelliferins on growth and alcoholic fermentation by a strain of \textit{Saccharomyces cerevisiae} and against 6 selected bacteria are described. The purpose of this study was to determine if the isolated flabelliferins have antimicrobial action.

**METHODS AND MATERIALS**

Palmyrah fruits were collected from Hambantota and pulp extracted manually. The isolation of flabelliferin was carried out as described previously.

**Yeast growth experiments**

\textit{Saccharomyces cerevisiae} strain S11-F3 was grown on YPD (Yeast Potato Dextrose) slant cultures and inoculated into a synthetic liquid medium with glucose 3% as carbohydrate source. After 24 h, 1ml of this culture was centrifuged at 25\(^\circ\)C. The yeast cells were inoculated into 50ml of the same medium in a 250ml flask and incubated at 37\(^\circ\)C. For growth studies an initial haemocytometer count was obtained. Growth was estimated spectrophotometrically at 660 nm at 0, 2, 4 and 6 h in flasks with 60 and 250 \( \mu \)g/ml flabelliferins which was added into the liquid culture prior to sterilization. Controls were used with the same amount of yeast but no flabelliferins.

**Effect of flabelliferins on fermentation**

For alcoholic fermentation the seed culture (25ml) was centrifuged at 25\(^\circ\)C in a bench centrifuge. After one day (log phase), the yeast was inoculated into a same medium as above but containing 20% glucose and fermented at 37\(^\circ\)C in a 250ml Erlenmeyer flask for three (3) days, with flabelliferins (1mg/ml) introduced as above. Controls were also used. The time course for fermentation was followed by measuring \( CO_2 \) evolved estimated by loss of weight of the fermentation flask. The fermentation flasks were fitted and weighed with fermentation bungs and U-tube containing conc. \( H_2SO_4 \) (which allowed only evolution of \( CO_2 \) from the system).

Alcohol was determined by the specific gravity method after distillation and residual sugar was determined by the Nelson method.
Effect of flabelliferins on bacterial growth

This was conducted using the following bacterial cultures *Staphylococcus aureus* (NCTC 8532), *Staphylococcus epidermidis* (NCTC 4276), *Escherichia coli* (NCTC 10148), *Pseudomonas aeruginosa* (NCTC 10662), *Proteus rettgeri* (NCTC 7475), *Acinetobacter calcoaceticus* (NCTC 5866).

They were cultured on nutrient agar slants and transferred to growth media containing nutrient agar broth for about 18 h and then spectrophotometrically checked (660 nm) to confirm extent of growth. An aliquot (0.1 ml) was spread on nutrient agar plates.

Nutrient-agar plates had the following composition: nutrient agar, 28 g l\(^{-1}\); agar, 2%. This was used to monitor the effect of flabelliferins (62.5 µg - 2500 µg) per filter paper disc (9 mm) using the Bauer-Kirby method.\(^{10}\) Each flabelliferin was dissolved in alcohol and dried on the 9 mm disc. Alcohol (dried with hot air) and ampicillin standard discs (33 µg) were used as controls and as standards. After 24 h, the inhibition zone diameters (in mm) were measured from replicates for each concentration.

RESULTS

Growth studies on yeast

Results are shown in Fig 1, 2, 3, and 4. Where F-II was found to inhibit growth 50-75% at 250 µg ml\(^{-1}\) and F\(_B\) completely at 60 µg ml\(^{-1}\). F\(_C\) & F\(_D\) did not inhibit growth at 250 µg ml\(^{-1}\). All experiments were done in duplicate.

Effect on alcoholic fermentation

Results (Figure 5, 6, 7 and 8) showed that once again F\(_B\) was most bioactive in producing a lag period in fermentation. It was observed (Table 1) that in all cases fermentation efficiency (conversion of glucose to alcohol taking into account residual sugar) was relatively unaffected (86-90%). A concentration of 1mg/ml flabelliferins was used for each flask. All experiments were done in duplicate.

Effect on bacterial growth

The crude extract F-II showed inhibition zones for only three bacteria while F\(_C\) and F\(_D\) did not inhibit the bacterial growth (Table 2). However F\(_H\) inhibited all the bacterial strains tested (Table 3).
Figure 1: Effect of F-II on growth of *Saccharomyces cerevisiae*.

Figure 2: Effect of F-II on growth of *Saccharomyces cerevisiae*.
Figure 3: Effect of $F_r$ on growth of *Saccharomyces cerevisiae*.

Figure 4: Effect of $F_b$ on growth of *Saccharomyces cerevisiae*. 
Figure 5: Effect of $F_a$ on alcoholic fermentation.

Figure 6: Effect of $F_{II}$ on alcoholic fermentation.
Figure 7: Effect of $F_e$ on alcoholic fermentation.

Figure 8: Effect of $F_o$ on alcoholic fermentation.
### Table 1: Summary of effect of flabelliferins on fermentation efficiency.

| Sample   | Alcohol (% w/v) | Residual sugar (%) | Efficiency (%) |
|----------|-----------------|--------------------|----------------|
| Control - 1 | 7.8             | 1.70               | 85             |
| Control - 2 | 7.7             | 2.67               | 89             |
| F-II - 1   | 5.5             | 6.53               | 86             |
| F-II - 2   | 6.6             | 4.69               | 86             |
| Control 1  | 7.9             | 2.62               | 90             |
| F_b - 1    | 6.6             | 4.40               | 90             |
| F_b - 2    | 7.0             | 3.80               | 86             |
| Control - 1 | 8.7             | Not detected       | 85             |
| Control - 2 | 9.4             | Not detected       | 92             |
| F_c - 1    | 9.4             | Not detected       | 91             |
| F_c - 2    | 8.8             | 0.06               | 86             |
| F_d - 1    | 8.9             | 0.09               | 87             |
| F_d - 2    | 9.0             | 0.13               | 89             |

### Table 2: Test for antibacterial action.

| Amount (mg) | Sample | Inhibition zone (mm) | Bacterial species                  |
|-------------|--------|----------------------|-----------------------------------|
| 12.5        | Crude  | 17.0                 | *Staphylococcus aureus*            |
|             |        |                      | *Staphylococcus epidermidis*       |
|             |        |                      | *Acinetobacter calcoaceticus*      |
| 2.5         | F-II   | No zone              | For all Bacteria                  |
| 2.5         | F_c    | No zone              | For all Bacteria                  |
| 2.5         | F_d    | No zone              | For all Bacteria                  |
Table 3: Anti-bacterial activity of $F_B$.

| $F_B$ amount (µg) | 1  | 2  | 3  | 4  | 5  | 6  |
|------------------|----|----|----|----|----|----|
| 2500             | 31.3 | 31.0 | 24.0 | 39.0 | 25.5 | 22.0 |
| 1250             | 29  | ND  | ND  | ND  | ND  | ND  |
| 625              | 23  | ND  | ND  | ND  | ND  | ND  |
| 312              | 18.7 | ND  | ND  | ND  | ND  | ND  |
| 167              | 21.1 | 13.5 | 14.5 | 14.7 | 14.5 | 14.5 |
| 125              | 21.2 | 17.0 | 13.5 | 12.5 | 12.5 | 13.5 |
| 62               | 15.9 | 14.5 | 12.0 | 13.0 | 12.0 | 13.0 |

Ampicillin (33 µg) → Inhibition zone 35 mm
ND - Not detected

#*# 1 *Staphylococcus aureus* - NCTC 8532
2 *Staphylococcus epidermidis* - NCTC 4276
3 *E. coli* - NCTC 10148
4 *Pseudomonas aeruginosa* - NCTC 10662
5 *Proteus rettgeri* - NCTC 7475
6 *Acinetobacter calcoaceticus* (var. lowffic) - NCTC 5866

#*# refers to numbers in table.

**DISCUSSION**

Results indicated that flabelliferin $F_B$, a steroidal triglycoside was an inhibitor of yeast growth, alcoholic fermentation and bacterial growth. This explains why palmyrah fruit pulp ferments very slowly and does not spoil easily. $F_B$ affected alcoholic fermentation by only extending the lag period. The results are interesting as $F_B$ was detected only in samples from Hambantota. Another interesting feature was that both $F_B$ (active) and $F_C$ (inactive) are isomers (M.W. 868). It is anticipated that elucidation of the detailed structured features of the carbohydrate moiety of $F_B$ and $F_C$ will provide insight into structure-bioactivity relationships. Other interesting follow up work include:

1. determination of the diversity of flabelliferins of different known morphological types of tree.

2. determination if any of the products of debittering correspond to $F_B$. 
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