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

11. Preliminary investigations of effect on yeast and selected bacteria

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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 F_B and F_C (triglycosides) and flabelliferin F_D (a diglycoside).

On testing for bioactivity, F-II, the bitter saponin (250 μg ml^{-1}) inhibited yeast (*Saccharomyces cerevisiae* S11- F_3) growth to the extent of 50 - 75% while F_B (60μg ml^{-1}) inhibited growth completely. F_C and F_D were inactive . F_B and F-II slowed the rate of alcoholic fermentation (F_B being most potent). But F_C and F_D 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 F_B was active inhibiting all bacteria tested, namely; *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa, Proteus rettigeri* and *Acinetobacter calcoceticus*. The study is of interest in the area of structure activity relationships, as F_B (active) and F_C (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 rhamnose 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 F_B, F_C, & F_D which were isolated by flash chromatography.[4]

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Flabelliferins F_B & F_C were triglycosides (M.W.868) and flabelliferin F_D a diglycoside (M.W.722).[4] F_B was found to be most foam stabilizing and haemolytic .[4] 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.[5]

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 *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.[3] The isolation of flabelliferin was carried out as described previously.[4]

Yeast growth experiments

Saccharomyces cerevisiae strain S11- F3 was grown on YPD (Yeast Potato Dextrose) slant cultures and inoculated into a synthetic liquid medium[6] with glucose 3% as carbohydrate source. After 24 h, 1ml of this culture was centrifuged at 25°C. The yeast cells were inoculated into 50ml of the same medium in a 250ml flask and incubated at 37° 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 µg/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°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°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.[7] 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[8] and residual sugar was determined by the Nelson method.[9]

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 rettigeri* (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 gl^{-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 9mm 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_B inhibited all the bacterial strains tested (Table 3).

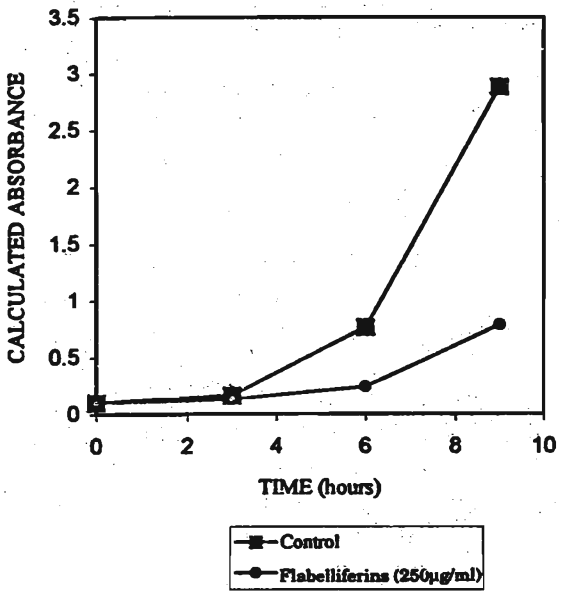

Figure 1: Effect of F-II on growth of *Saccharomyces cerevisiae*.

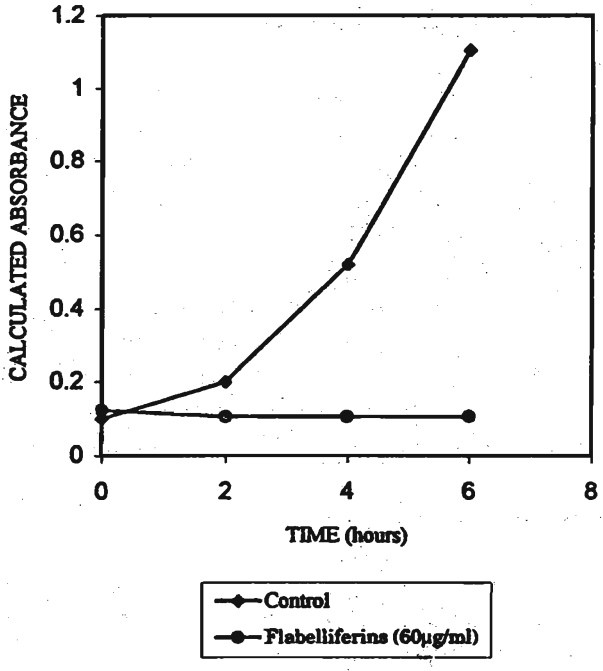

Figure 2: Effect of F_{II} on growth of *Saccharomyces cerevisiae*.

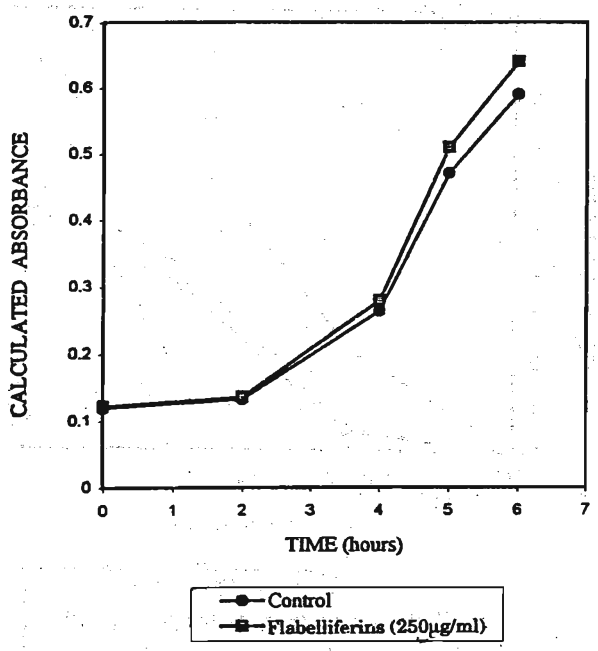

Figure 3: Effect of F_C on growth of *Saccharomyces cerevisiae*.

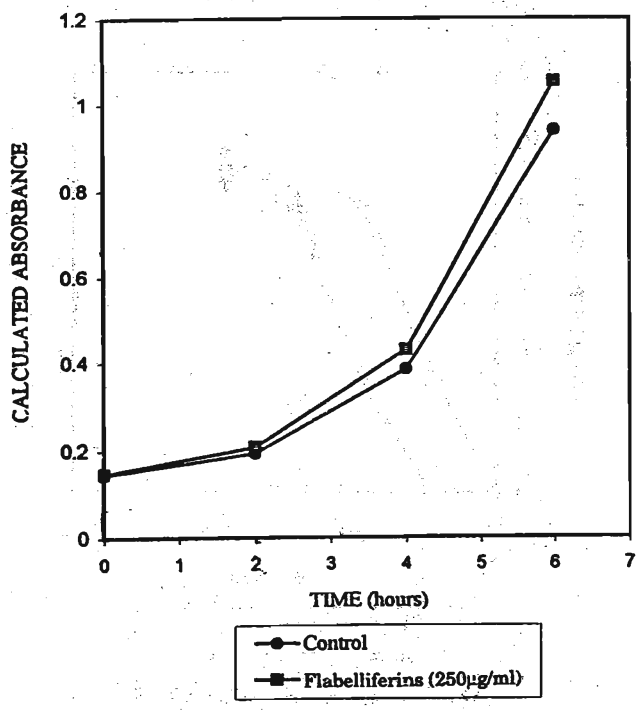

Figure 4: Effect of F_D on growth of *Saccharomyces cerevisiae*.

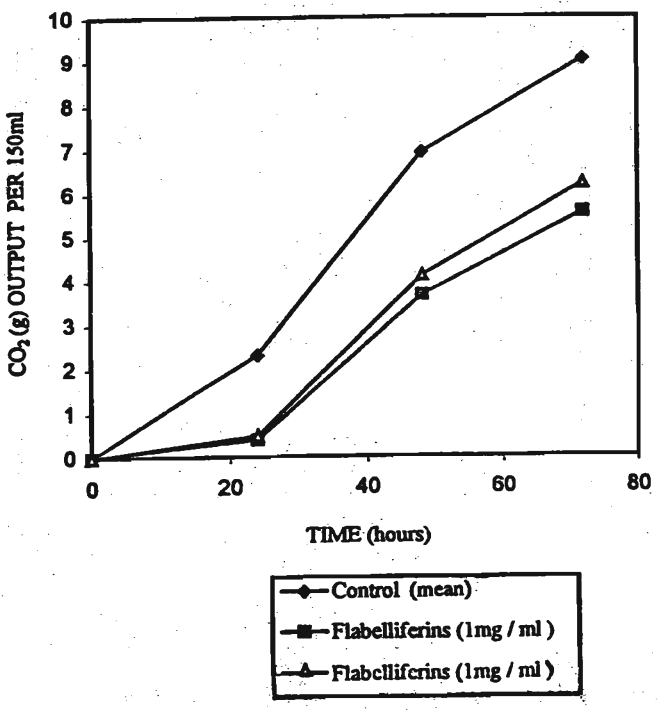

Figure 5: Effect of F_B on alcoholic fermentation.

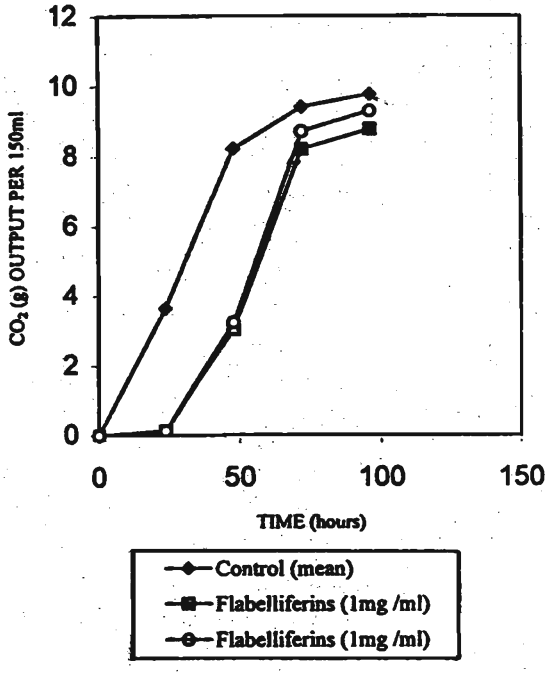

Figure 6: Effect of F-II on alcoholic fermentation.

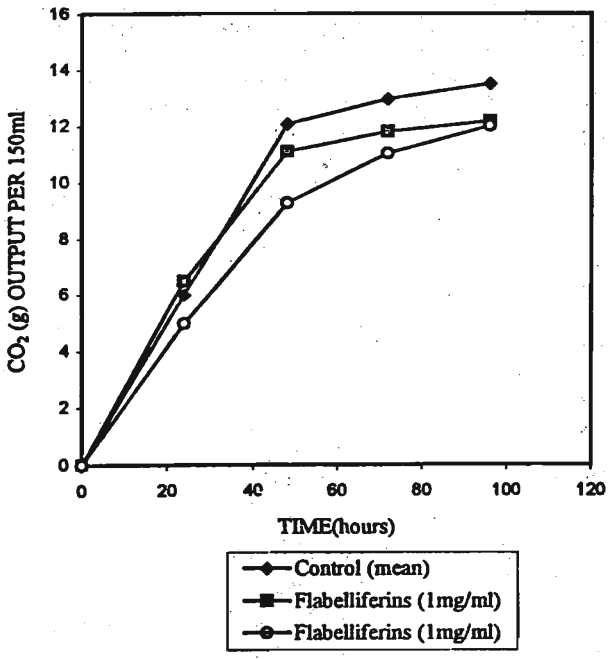

Figure 7: Effect of F_C on alcoholic fermentation.

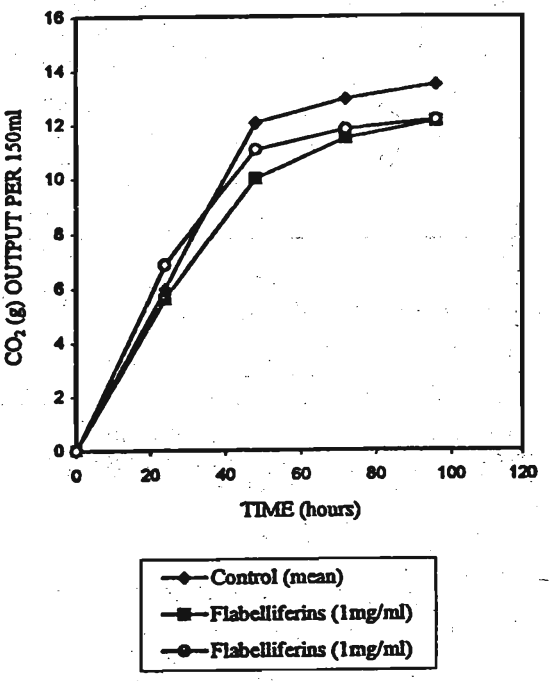

Figure 8: Effect of F_D on alcoholic fermentation.

Table 1: Summary of effect of flabelliferins on fermentation efficiency.

Sample	Alcohol (%) w/v	Residual sugar (%)	Efficiency (%)	
Control - 1	7.8	1.70	85	87
Control - 2	7.7	2.67	89	
F-II - 1	5.5	6.53	86	86
F-II - 2	6.6	4.69	86	
Control 1	7.9	2.62	90	
F_B - 1	6.6	4.40	90	88
F_B - 2	7.0	3.80	86	
Control - 1	8.7	Not detected	85	88
Control - 2	9.4	Not detected	92	
F_C - 1	9.4	Not detected	91	88
F_C - 2	8.8	0.06	86	
F_D - 1	8.9	0.09	87	88
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)	Inhibition zone (mm)					
**	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 rettigeri* - 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).[3] 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.

(11) determination if any of the products of debittering[3] correspond to F_B.

Acknowledgements

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