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Microcalorimetric investigations on the influence of propolis on the bacterium Micrococcus luteus[1]

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Abstract

Propolis or bee-glue is the third important product of honey bees after honey and wax. Known for thousands of years for its curative effects it is finding evermore attention in alternative and classical therapy in recent times against, e.g. infections, inflammations, dermatologic diseases and in wound healing.

Microcalorimetric experiments were performed on the influence of several propolis samples from Germany, Uruguay, Ethiopia and of a commercial propolis tincture on growth and metabolism of the recommended gram-positive bacterium *Micrococcus luteus*. Propolis extracts were prepared following established recipies for the water-soluble and insoluble components and the precipitate.

Addition of these extracts to a growing *M. luteus* culture in different growth phases resulted in a strong decline of the heat production rate, a prolongation of the lag phase or an introduction of a new, second lag phase, while the form of the calorimetric power-time curve remained unchanged. The calorimetric response showed a linear dependence on the propolis concentration. Although the quantitative gain of the extracts from the different propolis samples was nearly constant, the effects varied considerably between the specimens used.

The calorimetric investigations were supplemented by polarographic oxygen monitoring and by the standard agar well technique to determine the growth inhibition factor of the propolis extracts.

Resumé

Propolis est la troisième substance importante produit par les abeilles. Ses vertus curatives, bien que connues depuis des milliers d'années, ne retiennent l'attention de la medicine alternative occidentale aussi bien que de la medicine classique que depuis ces dernieres années dans le traitement des infections, des inflammations, des plaies et des maladies de la peau.

Ce travail relate les experiences de microcalorimétrie faites sur des échantillons de propolis provenant d'Allemagne, d'Uruguay et d'Ethiopie, ainsi que sur une teinture de propolis commercialisée, afin de determiner l'effet de ces échantillons sur la croissance et la métabolisme de la bacterie test Gram positive: *Micrococcus luteus*. Les extraits de propolis utilisés (partie soluble et insoluble dans l'eau ainsi que le sédiment) ont été obtenus à l'aide de procédes d'usage.

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[1]Dedicated to Professor Dr. Pierre Boivinet on the occasion of his 75th birthday.

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L'addition des ces extraits à differents stades de la croissance d'une culture de *M. luteus* conduit à une fort diminution de la production de chaleur, à une augmentation de la phase de latence ou bien à l'introduction d'une autre phase de latence alors que la forme des courbes calorimétriques reste inchangée. La response calorimétrique est proportionelle à la concentration de propolis. Bien que la quantité d'extrait obtenue à partir des echantillons soit presque constante, les effects observés varient très fortement.

Les mesures calorimétriques ont été complétées par des mesures polarographiques pour déterminer l'utilisation de l'oxygène et par la méthode classique de la zone d'inhibition de la croissance bactérienne par les divers extraits de propolis.

I.L. dédie ce travail à monsieur le Professeur Dr. P. Boivinet/Caen en témoignage de sa reconnaissance et du grand respect qu'il lui porte à l'occasion de son 75ième anniversaire et le prie d'accepter l'expression de ses sincères salutations.

Keywords: Honey bees; Metabolism; Microcalorimetry; *Micrococcus luteus*; Propolis

1. Introduction

Propolis or bee-glue is a resinous natural product like wax and honey gathered by honey bees and is accumulated in the beehive [1]. Investigations on the origin of propolis showed that bees collect propolis from resinous buds of a large variety of trees [2]. Only in few cases chemical analyses have been performed to find out the proposed relation between certain trees and their propolis samples [3]. In temperate zones, poplar buds appeared to be the main source of propolis, especially *Poplus nigra* L. [4–7] followed by birch (see Fig. 1), oak, alder, willow, and hazel in Europe, the *Acacia karoo* tree in South Africa, the grass tree *Xanthorrhoea pressi* and *X. australis* in Australia, *Plumeria accuminata*, *P. acustifolia* and *Psidium guajava* in North America [8]. However, nearly nothing is known on the sources of propolis in the tropics [9]. Investigations on propolis from Venezuela [9] and Brazil [10] showed absence of poplar polyphenols which are the chief constituents of temperate propolis. Propolis samples differ substantially in their chemical composition, depending on the geographical location of the collection site [8], but also between beehives in the same place when bees do not have equal access to the same plants.

Despite this chemical variation the basic makeup is usually maintained as 55% resin, 30% wax, 10% essential oils and 5% pollen [11]. Resin comprises varying amounts of chemicals from plants and glandular secretions of worker bees. It can therefore be asserted that the chemical composition of propolis is very complex; and more than 160 constituents have been identified so far from propolis of different geographical origins [3]. Propolis varies in colour from light brown to a dark chestnut, depending on its source and age, and has a strong aroma. Freshly collected propolis is very sticky at the normal temperature of hives (~35°C), becoming quite hard below 15°C and brittle below 5°C [8].

Propolis is gathered by honey bees from leaf buds, breaks in the bark of trees and coating of pollen grains. Worker bees in the beehive then add glandular secretions, such as enzymes and waxes, to propolis [5]. No further changes in the chemical composition of propolis could be ascertained [8].

Propolis is produced by trees to avoid infection of injured tree parts, retard bud development during frost, and protect pollen from being infected by microbes [12].

Honey bees use propolis to lute leakages in the hive and to varnish all the inside surfaces of the hive. A thin layer of propolis on the inner wall of the beehive impedes loss of moisture and hence maintains a moist and warm condition for the developing brood [13]. Moreover, the propolis layer prevents the growth of bacteria and mould under these otherwise favourable conditions [14].

Intruders too large to be moved out from the hive are killed and encased in propolis to prevent rotting. The embalmment and thus slow mummification of intruders may serve to contain putrefaction, and thus the spread of infection and disease [8].

The medical value of propolis for the honey bees is equally beneficial to humans. Propolis has been used in folk medicine since the time of the Pharaohs [15]. There is evidence that in prehistoric times, surgery was performed with propolis as an antiseptic [16]. Nowadays, propolis has attracted attention as a general natural cure as well as for some clinical applications due to the development of drug resistance by

DISPENSA-
TORIVM
PHARMACORVM
omnium, quæ in uſu potiſsi-
mum ſunt.

Ex optimis AVTORIBVS, tàm recentibus quàm veteribus, collectum, ac ſcholiis utilibus illuſtratum, in quibus imprimis ſimplicia diligenter explicantur.

AVTORE PRIMO
VALERIO CORDO
Nunc cum aliis pluribus ad hanc rem pertinentibus, quæ ſequentibus indicantur.

OPERA ET STVDIO
COLLEGII MEDICI INCLYTÆ REIPVB. NORIBERGENSIS, multo emendatius ac ſelectis compoſitionibus auctius, ex ſecunda EDITIONE publicatum.

Cum copioſo duplici Indice: & Privilegio Imperiali.

NORIBERGÆ,
Excudebat Paulus Kaufmann.
M. D. XCVIII.

Fiat Emplaſtrum ſecundum artem ac diligenter.

PROPOLIS *non ſunt ſordes alvearium apum, ſed eſt cera odorata, qua apes ante hyemem foramina alvearium obturant. Germanice vocatur* Bet & Methwachß, *eam apes colligunt ex oculis populi & betulæ arborum.*

VISCVM *Quercinum non hic ſignificat lignum viſci quercini, ſed gluten illud lentum ex viſco quercino extractum, quo capiuntur aves.*

SQVAMA *Æris duplex eſt, una craſſa, germanicè* Kupfferſchlag | *altera farinæ modo tenuis & ruffa* Keſſelbraun | *eſtque efficacior, atque ideo hic aſſumi debet.*

Valet ad dolores cervicis potiſs. & renum. Sagittas & alia corpori infixa extrahit.

Fig. 1. Title page (left) and enlarged section of page 199 (right) concerning propolis of the Pharmacopeia of Valerius Cordus (1598).

pathogenic microbes and the widespread occurrence of disfunction of the immune system [17]. Immunomodulation through natural or synthetic substances may be considered an alternative for the prevention and cure of infectious diseases [18–20]. Propolis possesses versatile biological activities including antibacterial, antiviral, antifungal, immunostimulating, hypotensive, cytostatic [8], antiinflammatory [21] and antiulcerous [22]. Most effects are still based on the excellent wound healing and anti-inflammatory properties of propolis [23]. But, besides these positive influences propolis may also have severe drawbacks in the form of allergic reactions [24–26].

Though the antimicrobial activities of propolis and some of its constituent compounds, especially the flavonoids, have widely been reported, its mechanism of action (bactericidal/bacteriostatic) has not yet been clearly understood. Since propolis extracts are coloured and turbid mixtures, it is difficult to investigate their antibacterial activity by a simple turbidity test (incubation in liquid medium and examining the evidence of growth by eye or photometry). Calorimetry as a non-specific, integrating tool is a suitable method to investigate the action of propolis on bacterial growth and metabolism.

These flow-calorimetric investigations on metabolism and growth of the chosen bacterium were supplemented by oxygen polarography and the classical agar well diffusion. Whereas the latter renders only end point figures of growth inhibition, calorimetry and polarography are on-line techniques and respond to the kinetics of the processes as well. But together, they all lead to a complex picture of the actions of different propolis preparations.

2. Materials and methods

2.1. Biological material

All experiments were performed with the gram-positive bacterium *Micrococcus luteus* (strain 348, ATCC 9341). The sample was a kind gift from Prof. Dr. Gunda Kraepelin, Technical University of Berlin, Institute of Microbiology. The optimal temperature of growth was determined at 30°C, the optical density (OD) at 646 nm as $(0.434)^{-1} \times 10^8$ cells ml^{-1}.

The liquid growth medium was composed of peptone (10 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.247 g l^{-1}), and NaCl (5 g l^{-1}) in deionized water. Solid growth medium was prepared by addition of 15 g agar to 1 l of liquid medium. The pH of the growth medium was adjusted to 7.0 by using 1 N NaOH and/or 1 N HCl prior to sterilization (15 min at 121°C). Stock cultures were kept on solid agar slants at 4°C. One day before each experiment, cells were inoculated into a 50 ml Erlenmeyer flask containing 20 ml liquid medium and incubated at 30°C on rotary shaker at a stirring rate of 125 rpm.

2.2. Extraction of propolis

Propolis samples of different geographical origin were applied in these investigations. They include specimens from Germany (prop. 1) and Uruguay (prop. 2) both obtained as a gift from the Pharmaceutical Institute of the Free University of Berlin, Ethiopian propolis (prop. 3) collected from Holleta Bee Research Centre and a commercial Aagaard propolis tincture of German origin (10% in 60% ethanol; Börner GmbH, Berlin, Germany). This tincture is on the market as a medicinal agent for the treatment of upper respiratory tract infections.

The solid propolis samples were homogenized by a coffee mill (type MZ, Moulinex, France). The ground powder was suspended in 70% methanol [27] in a ratio of 10% (w/v) for effective extraction [28] in a rotary evaporator (Rotations-Verdampfer W-micro, Heidolph, Mannheim, Germany) for 2 h at 60°C. After that, the suspension was suction-filtered using a filter paper (Selecta Filter NR 595 1/2) [29] and dried in an oven at 40°C to weight constancy. The yield of extraction was approximately 21% (w/w) for all propolis samples used. The powdered form of the extract was dissolved in 60% ethanol to make a 10% (w/v) stock solution and stored in a refrigerator for later use.

The ethanol solution of unprecipitated propolis possesses both water-soluble and insoluble components. Addition of this propolis solution to the growth medium leads to the formation of a white precipitate, a problem already mentioned by other authors [30,31]. To separate the water-insoluble components of propolis, 1 ml of the stock solution was mixed with 9 ml distilled water. The precipitate was separated by centrifugation at $4500 \times g$ for 50 min [29]. The precipitate fully dissolved in 60% ethanol and maintained the colour of the original propolis solution. The water-soluble component however, had a colour of whitish-grey for all propolis samples. The water-soluble and -insoluble components, both in solution, were dried in an oven at 40°C and afterwards dissolved again in the appropriate solvent (distilled H_2O or ethanol) to make 10% stock solutions. Calorimetric comparison of the activities of the different preparations (water-soluble, -insoluble and unprecipitated) was done at a concentration of 1 mg ml^{-1}.

2.3. Calorimetry

A flow calorimeter (type 10700-1, LKB, Bromma, Sweden) with a flow-through vessel of 0.587 ml was used for all calorimetric investigations at a temperature of 30°C. The calorimeter was connected by a peristaltic pump (LKB, Pharmacia, Bromma, Sweden) and a Teflon tube of ∅ 0.1 cm to a separate fermenter in a water bath also controlled at 30°C. The connection line between the calorimeter and the fermenter could house different electrodes for a simultaneous determination of heat production rate, oxygen consumption rate and pH. In the present work an oxygen electrode (Eschweiler, Kiel, Germany) was incorporated directly after the calorimeter to determine the oxygen concentration in the outflowing medium.

To satisfy the requirement for both a high flow rate (to avoid settling of cells and exhaustion of substrates during the residence time in the tubing system) and a slow one (to allow sufficient temperature equilibration and to avoid heat creation by friction), a pumping rate of 100 ml h^{-1} was used for investigations with a prevailing aerobic metabolism [32]. At this pumping rate a sample needed 1.42 min

to reach the calorimetric vessel and 3.0 min to return to the fermenter.

The calorimetric signal was amplified and recorded as power–time curve (heat production rate P vs. time t) by a two-channel recorder (BD5, Kipp and Zonen) at a full scale deflection of 50 μV and a chart speed of 0.02 cm min^{-1}.

2.4. Sterilization and cleaning of the flow line

Since cells of *M. luteus* grow on the inner walls of the vessel and the flow lines, intensive cleaning and sterilization was necessary before and after each experiment. A solution of 10% EtOH and 2% H_2SO_4 in distilled water [32] was pumped through the flow line and allowed to act for at least 20 min. After this, the tubes were rinsed by a sterile solution of KH_2PO_4 buffer at pH 7.0 for at least 30 min and the sterile growth medium was pumped through the whole system until a stable baseline was obtained. The cell suspension was then added to establish a P–t curve.

Addition of propolis to the medium leads to a precipitation of the water-insoluble components of propolis on the inner walls of vessel and line. Therefore, a special solution of 10% H_2O_2 and 2% H_2SO_4 in 60% ethanol was applied for dissolving propolis and lysing the bacterial cells to get the line clean and sterile. The routine washing with the buffer was carried out as already described.

2.5. Polarographic oxygen sensors

It has been suggested [33] that oxygen becomes a limiting factor in the flow line at higher cell densities, elevated substrate concentrations and/or reduced pumping rates. Because of this a polarographic oxygen sensor (type EaKl, Eschweiler, Kiel, Germany) was incorporated into the flow line after the calorimeter. The signal from the sensor was recorded by a two-channel recorder (type LS4, Linseis GmbH, Selb, Germany) at a full scale deflection of 20 mV and a chart speed of 10 mm h^{-1}.

In order to ascertain the physiological state of the culture, the oxygen consumption rate of the cell suspension at 30°C was measured in parallel with the flow calorimetric experiments by a separate batch oxygen electrode (Bachofer, Reutlingen, Germany). Aliquots of 1 ml were transferred from the outlet of the flow line to the electrode at given times during growth. They were aerated and the signals recorded by a two-channel recorder (Servogor XY 743; Goerz, Austria) at a full scale deflection of 20 mV and chart speed of 3 cm min^{-1}. Calibration of the electrodes was performed every day by established methods [34].

Polarographic experiments for treatments with unprecipitated alcoholic extracts were not possible due to precipitation of the water-insoluble components on the surface of the membrane and hence blockage of the diffusion of oxygen to the electrode. Similarly, the tubing system in the oxygen sensor incorporated in the flow line was completely blocked by precipitation. Due to these problems antibacterial activity of propolis was investigated polarographically only in a few cases.

2.6. Agar well diffusion assay

In addition to calorimetry, the antibacterial activity of propolis was investigated by the agar well diffusion assay method. Sterile growth medium was poured into sterile petri dishes to render a 3 mm thick agar layer. To achieve a lawn of grown cells, 0.5 ml of cell suspension (OD 0.8 at 646 nm) were evenly spread on the agar surface. After 1 h, wells were cut into the agar with a flame-sterilized and cooled cork borer of ∅ 8 mm. Each plate housed four wells for fourfold determinations. The wells were filled with 0.1 ml of a given extract concentration. Blanks of 60% ethanol or distilled water were tested as control. The latter blank was also useful for the water-soluble components of propolis. In every case each concentration was monitored fourfold.

The plates were incubated for 48 h at 30°C and displayed on a microfiche screen with a magnification of 9.8× to determine the radius of the clear zone. Antibacterial activity was then expressed as the radius of the clear zone (mm).

3. Results

3.1. Standard curve

Microscopic examinations of cell morphology were done before and after the treatment with different extracts, taking samples at the outlet of the flow line.

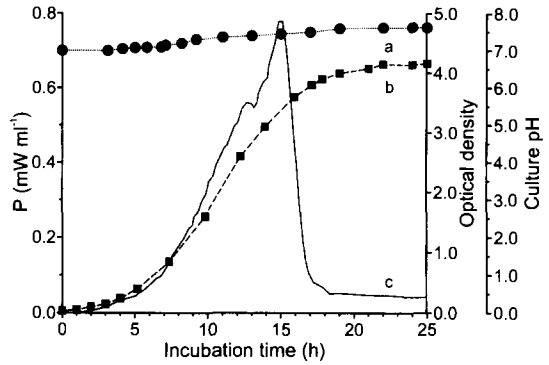

Fig. 2. Typical ('standard') growth curve of *M. luteus* in a complex medium. (a): pH of the culture; (b): Optical density; (c): Flow calorimetric power–time curve.

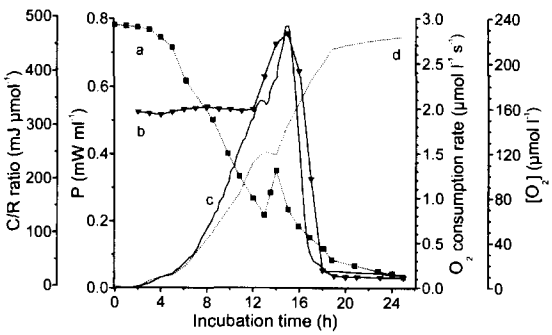

Fig. 3. Calorimetric curve (c) as shown in Fig. 2 together with some further details. (a): Oxygen concentration in the flow line; (b): Calorespirometric (*C/R*) ratio; (c): Power–time curve; and (d): Oxygen consumption rate in the fermenter, as functions of time.

Before treatment, three or more cells stuck together and rarely occurred as single cells. After treatment, however, cells were mainly found as single cells indicating that the antimicrobial agents affected the cell wall. Treatment with 60% ethanol resulted in no visible change on the appearance of cells (see Fig. 1).

Fig. 2 shows the typical growth of *M. luteus* in the complex growth medium. The shape of the power–time (*P–t*) curve did not change when the inoculum was varied between $(0.078–2.4)\times10^7$ cells ml^{-1} except for the length of the lag phase (not shown). The total heat produced up to the return to the low level of metabolism was constant at 17.7 ± 0.4 J ml^{-1} with a constant final cell count of $(6.6\pm0.2)\times10^8$ cells ml^{-1} independent of the initial inoculum. The corresponding final optical density amounted to 4.2; the pH slightly increased during the exponential

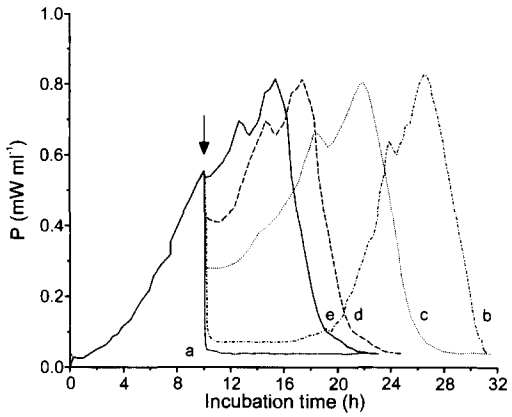

Fig. 4. Effect of propolis tincture added in the mid-exponential phase (arrow) of a *M. luteus* culture. The final tincture concentrations were as follows: (a): 1.0; (b): 0.8; (c): 0.6; (d): 0.3; and (e): 0.15 mg ml^{-1}.

growth phase by 0.6. These results were taken as 'standard' curves for all following investigations.

Some more details of the undisturbed growth of *M. luteus* can be found in Fig. 3. Besides the *P–t* curve (c) of Fig. 2, the oxygen consumption rate in the fermenter (d), the oxygen concentration in the flow line directly after the calorimetric vessel (a) and the calorespiratory coefficient *C/R* (b) are presented. The latter remains constant during the main part of the exponential phase and increases significantly due to strong contributions of anaerobic metabolism. The steep decrease after the main peak has to be considered as an artifact due to the exhaustion of oxygen in the flow line [33], while the oxygen consumption rate still increases in the fermenter.

From both, *P–t* and optical density curves, the heat production rate per single cell can be calculated. It slowly rises in the first 4 h from a very small figure of 0.05×10^{-12} W $cell^{-1}$; in the fifth hour when there is a rapid increase to 1.1×10^{-12} W $cell^{-1}$, a value which remains constant throughout the whole exponential phase. With the drop after the main peak, it returns to the initial value of 0.04×10^{-12} W $cell^{-1}$ mainly due to the exhaustion of oxygen in the flow line (not shown).

3.2. *Influence of propolis*

The effect of commercial propolis tincture on *M. Luteus* was tested in different growth phases. When

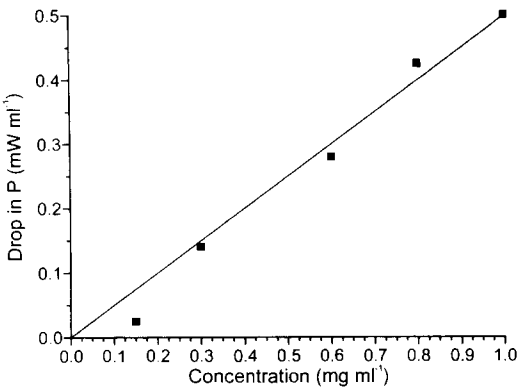

Fig. 5. Linear dependence of the drop in the heat production rate P after addition of tincture on the final concentration of tincture. Data taken from Fig. 4.

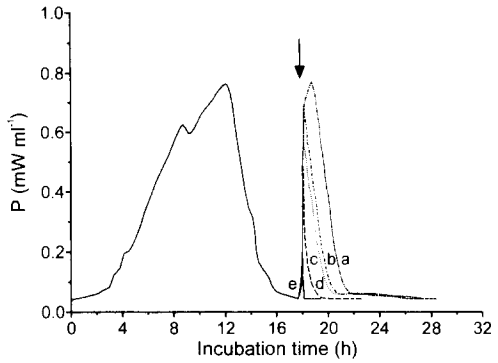

Fig. 7. Effect of propolis tincture (arrow) on the heat production rate at the end of the calorimetric growth curve. The applied concentrations are as in Fig. 4.

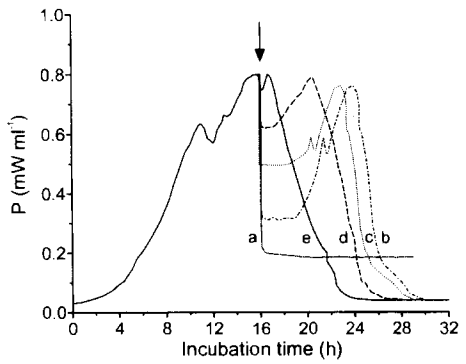

Fig. 6. Addition of propolis tincture (arrow) directly after the main peak of the standard growth curve. The applied concentrations are as in Fig. 4.

propolis was added in the late lag phase (5 h) it produced a small retardation at a concentration of 0.15 mg ml^{-1}. At all other concentrations the heat production rate dropped to nearly zero and did not recover during the next 6 h at 0.3 mg ml^{-1} and during the next 15 h at 0.6 mg ml^{-1}. In total, the effect resembles that of a prolonged lag phase due to decreased inoculum. It can be interpreted that only very few cells survived the attack of propolis and had to pass many generations till the cell number was high enough to be detected by calorimetric means (not shown).

In Fig. 4 the effect of propolis addition in the mid-exponential phase is presented. With increasing propolis concentrations the main peak is shifted to later times and the drop in heat production rate becomes more pronounced while the typical shape of the growth curve remains unchanged. Curve 'a' does not show any sign of new growth during the experimental period. Fig. 5 renders the linear dependence of the reduction in the P–t signal due to propolis and its concentration with a correlation coefficient of r^2=0.994. All curves return to the same minimum metabolic level as seen in the standard curve. Similar results are obtained when adding tincture directly after the main peak (Fig. 6). The absolute drop in heat production rate is nearly the same as in the mid-exponential phase but the relative one is smaller so that the resumption of growth occurs much earlier. Moreover, when the drop is large enough to fall below the level of the first smaller peak (see standard curve Fig. 2), this peak appears again for a second time. As in Fig. 4 no growth is seen at the highest tincture concentration within the experimental period while the heat production rate rests on a relatively high level, an indication that bacteriocidic and bacteriostatic actions work together, leading to the reduced metabolism of maintenance.

Addition of tincture some hours after the main peak shows an interesting effect: as opposed to the results in the preceding figures, the heat production increases dramatically (Fig. 7), followed by a more or less rapid return to the former low metabolism. Again a linear dependence between the increase in power and the propolis concentration is obvious. This on the first glance inexplicable result may be due to effects described by Motzkus et al. [32] and Hölzel et al.

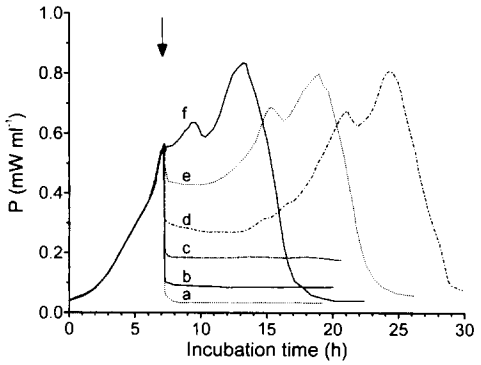

Fig. 8. Effect of the alcoholic extract of prop. 2 (Uruguay) on the standard growth curve of *M. luteus* at the following final concentrations: (a): 1.0; (b): 0.8; (c): 0.6; (d): 0.3; (e): 0.15; and (f): 0.072 mg ml^{-1}.

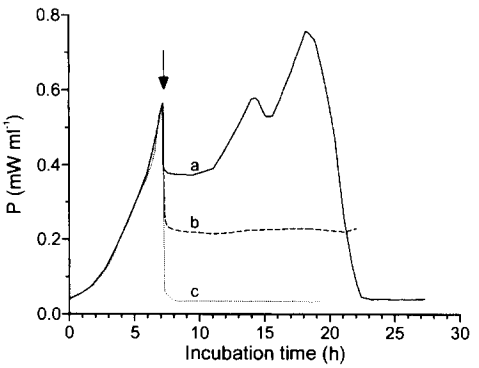

Fig. 10. Comparison of the effects of (a): water soluble; (b): water insoluble; and (c): alcoholic propolis extracts (Uruguay, prop. 2) on the standard growth curve of *M. luteus* after addition in the mid exponential phase (arrow).

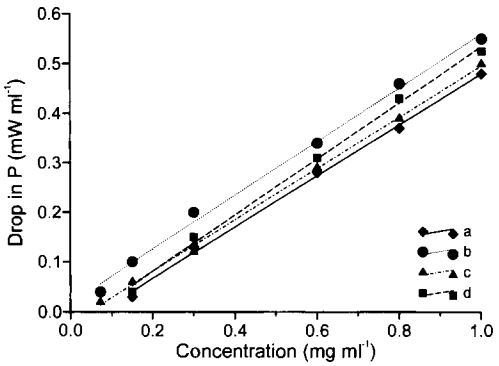

Fig. 9. Dose–response curves for the drop in the heat production rate *P* after addition of alcoholic extracts of different propolis samples in the mid-exponential phase. (a): from Germany (prop. 1); (b): from Uruguay (prop. 2); (c): from Ethiopia (prop. 3); (d): commercial tincture (for comparison).

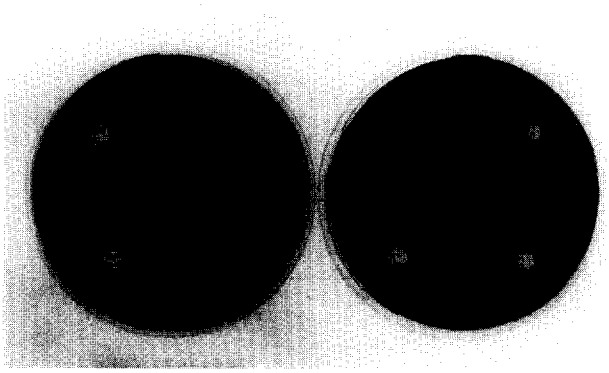

Fig. 11. Photograph of two petri dishes with four wells each in solid growth medium. Left: Tincture after precipitation of the water-insoluble components and Right: original tincture. Differently sized clear zones were produced by the indicated tincture concentrations. The dark spot in the upper left well of the right petri dish is due to precipitation of propolis. Some precipitation is also seen for 2%.

[33] for metabolic kinetics coming up by the combination of fermenter and flow calorimeter.

3.3. *Different preparations*

In the further experiments, different propolis samples, different preparations (water-soluble, water-insoluble and unprecipitated alcoholic extracts) and different final concentrations were compared concerning their influence on the standard growth curve of *M. luteus*. Representative results are shown in the following figures while general conclusions can be drawn for all samples.

Fig. 8 presents the influence on the power–time curves by an unprecipitated alcoholic extract of Uruguayan propolis (prop. 2) with decreasing concentrations (curves 'a'–'f'). The three highest concentrations are so active that growth was not resumed in the following 24 h. Concentration 'a' with 1.0 mg ml^{-1} even blocks to a high degree the metabolism of maintenance, whereas for the subsequent concentrations, the bacteriostatic action of propolis becomes visible. Fig. 8 resembles Fig. 4 for similar investigations with the commercial propolis tincture.

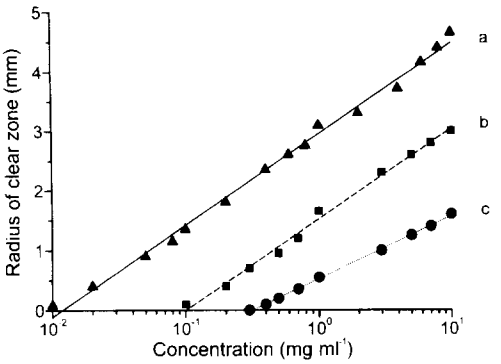

Fig. 12. Agar well diffusion assay. Dose–response curves of (a): alcoholic; (b): water insoluble and (c): water soluble extracts of propolis from Uruguay (prop. 2).

Trace 'e' still shows some inhibiting influences while trace 'f' is nearly identical with the standard growth curve. Thus, the minimal inhibitory concentration (MIC) of this extract can be assumed to be 0.072 mg ml^{-1}. The same MIC value was obtained for the Ethiopian extract and a figure of 0.15 mg ml^{-1} for the German one. Fig. 9 compares the drop in the power–time curves of the different extracts in the form of dose–response curves. It shows linear dependence on propolis concentration in the following order of decreasing influence: Uruguayan propolis > commercial tincture > Ethiopian propolis > German propolis. The observed differences are small.

Water-soluble, water-insoluble and unprecipitated alcoholic extracts exhibit increasing influences on the calorimetric growth curves in this order (Fig. 10). Whereas the water-soluble components only lead to a small drop in the heat production rate and a short additional lag phase, the two other extracts render their bacteriostatic/bacteriocidic activities with no further growth during the next 24 h. The drop in the power output achieved by the unprecipitated alcoholic extract was even higher than that of the sum of the other two. These results for Uruguayan propolis are similar to those for samples of German and Ethiopian origin.

The calorimetric experiments were supplemented by agar diffusion investigations on propolis inhibition of *M. luteus* growth (Fig. 11). The clear zones without visible growth around the wells containing propolis extracts showed a linear response on increasing concentrations (Fig. 12). Maximum radii amounted to 4.2–4.6 mm for unprecipitated alcoholic extracts. Fig. 12 resembles the result found in calorimetric growth curves (Fig. 10), showing that the water-soluble extract has the lowest effect and the water-insoluble one gives medium inhibition. Together, both are less than or just as effective as the alcoholic extract. The MIC values found with this technique are lower than the calorimetric ones.

4. Discussion

The antimicrobial and germicidal action of propolis was known since the days of the Pharaohs [15,35,36]. This knowledge was kept in East European countries, in parts of Asia and Africa but was lost in Western Europe after medieval times ([4]; Fig. 1). Only in recent decades the importance of this bee product was discovered again and scientific investigations started for different reasons. Intensive research with modern chemical techniques went for the constituents of propolis samples [3,8,10,27,37–40] and a manifold of microbial strains was tested in their sensitivity against propolis or propolis components (e.g. [8,41,42]). It was found out that the mixture of organic compounds in propolis is active against very different diseases (e.g. [8,43,44]) so that a broad spectrum of applications exists for propolis although the modes of action are not well-understood.

M. luteus was chosen for the present work because it is not pathogenic and has its growth optimum around 30°C. This organism was not reported to be resistant to any sort of antibiotics and is hence recommended as test organism [45].

Propolis samples from different geographical origins (Germany, Uruguay and Ethiopia) gave comparable yields of 21% upon extraction with 70% methanol. It is well known that propolis samples collected from regions of various climates and vegetation are differing in their chemical composition but show the same basic makeup: 55% resin, 30% wax, 10% essential oils and 5% pollen [11]. As a result, the percentage of alcohol extractable compounds remains constant. Unprecipitated alcohol extract possesses both water-soluble and insoluble compounds. The percentage of either of these two components varies insofar as the plant source of propolis is not the same and hence their different activities on *M. luteus*.

The presence of water-insoluble components in propolis introduces more difficulties in its clinical applications [17], especially as a drug to be administered orally. As an alternative, water-soluble derivative of propolis was prepared [46]. But it becomes clear from both calorimetry (Fig. 10) and agar well diffusion assay (Fig. 11) that the water-soluble components were inferior in activity to the other two preparations and that the full potency of propolis is not met when using only the water-soluble part of propolis.

Modern calorimetry of microorganisms proceeds in two different ways: (a) as a quantitative tool to evaluate thermodynamic quantities, determine metabolic pathways and establish energy balances [47–49]; and (b) as an analytical tool to prove the existence of microbial contaminations, characterize microorganisms by their power–time curves and evaluate the influence of drugs or xenobiotic compounds on growth and metabolism of cell cultures [50–54]. Here, calorimetry was applied in an analytical way to demonstrate the influence of different kinds of propolis extracts on metabolism and growth of *M. luteus*. The observed undisturbed heat production rates and the form of the ('standard') power–time curves correspond well with results found in the literature [47,49]. Until now only one further microcalorimetric paper on propolis exists, concerning the action of an unprecipitated alcoholic extract on the gram-positive bacterium *Streptococcus agalactiae* [55]. The authors observed the same drop in the heat production rate and the same long additional lag ('plateau') phase after the addition of extract in the mid-exponential phase as shown in Fig. 4 of this paper.

This drop and the plateau on varying levels indicate that the propolis extracts exhibit both, a bacteriostatic and a bacteriocidic action. It is well known that the metabolism of maintenance is lower than that of growth so that the reduced heat dissipation proves that microbial cells were inactivated concerning multiplication, but were still capable of intensive metabolism. At low extract concentrations in the medium, there is a good correlation between length of the additional lag phase and drop in heat production rate. It shows that the number of the non-inactivated growing cells is too small to be detected by the calorimeter and to render an increase in heat dissipation.

Calorimetry usually is an easy and effective tool to investigate the action of drugs on biological systems since it is a non-specific and integrative approach towards all heat producing processes and monitors aerobic as well as anaerobic metabolism [51]. In the case of propolis some problems arise due to the precipitation of some of the components in the aqueous medium and to clotting along the flow lines or in the calorimetric vessel. Thus, only low propolis concentrations can be investigated in a proper way. Nevertheless, the results presented here indicate that flow calorimeter/fermenter combinations are well suited for a quick screening of propolis extracts from different origins in their influence on some non-pathogenic and various pathogenic microorganisms of human importance. Perhaps, such investigations can help to clarify the mode of action of this bee product and to find new promising applications of this old remedy from Pharaoh times.

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