

Distinct antimicrobial activities in aphid galls on *Pistacia atlantica*

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Gall-formers are parasitic organisms that manipulate plant traits for their own benefit. Galls have been shown to protect their inhabitants from natural enemies such as predators and parasitoids by various chemical and mechanical means. Little attention, however, has been given to the possibility of defense against microbial pathogens in the humid and nutrient-rich gall environment. We found that the large, cauliflower-shaped, galls induced by the aphid *Slavum wertheimae* on buds of *Pistacia atlantica* trees express antibacterial and antifungal activities distinct from those found in leaves. Antibacterial activity was especially profound against *Bacillus* spp (a genus of many known insect pathogen) and against *Pseudomonas aeruginosa* (a known plant pathogen). Antifungal activity was also demonstrated against multiple filamentous fungi. Our results provide evidence for the protective antimicrobial role of galls. This remarkable antibacterial and antifungal activity in the galls of *S. wertheimae* may be of agricultural and pharmaceutical value.

Introduction

Galling habits have evolved convergently among and within numerous insect lineages indicating that the phenomenon is highly adaptive. Galls are formed by manipulation of plant tissues by gall-forming insects to their own benefit.^{1,2} Galls provide their inhabitants with better nutrition and protection from harsh abiotic conditions such as temperature, precipitation and radiation. Galls also provide defense against natural enemies including predators and parasitoids.^{1,3-5} Pathogens may be an important mortality agent of galling insect.^{6,7} Fungi for example, which were isolated from galls, were recognized as a main indirect source of mortality of gall-forming insects as they destroy gall tissues.⁸⁻¹¹ Accordingly, galled tissue was found to contain higher levels of defensive secondary metabolites compared with ungalled plant tissues.^{12,13} It is reasonable to expect that accumulation of such metabolites in the galls would provide multilevel protection against variable natural enemies, but its role in protection against microbial pathogens received little attention.

In the Levant, leaves and buds of wild pistachio (*Pistacia* spp) trees and shrubs serve as hosts for several gall-forming aphid species (Homoptera: Fordinae).¹⁴ The galls are long-lasting (from spring to fall), and in some species support up to thousands of aphids. Leaves of plants of the genus *Pistacia*, and especially their aphid-induced galls, contain high levels of secondary metabolites such as mono- and sesquiterpenes and pathogenesis-related (PR) proteins.¹⁵⁻¹⁷

In the present study, we focus on the gall-forming aphid *Slavum wertheimae*, which produces large cauliflower-shaped

galls (Fig. 1) on the lateral buds of *Pistacia atlantica* trees.⁵ These sealed galls may contain up to thousands of aphids for several months. The high humidity and availability of carbohydrates (including aphids' secretions) inside the gall should make the aphids highly vulnerable to microbial and fungal infection. We examined and compared the activities of *S. wertheimae* galls and ungalled *Pistacia atlantica* leaf tissues against various bacteria and fungi.

Results

Antibacterial activity. Since no specific bacterial pathogens of *S. wertheimae* or *Pistacia atlantica* are known, we tested the effect of galls and leaf powder aqueous extract on representative bacteria including known plant (*Pseudomonas aeruginosa*),^{18,19} and insect (*Bacillus* spp) pathogens.²⁰ Both green and red galls but not leaf extracts demonstrated antibacterial activity against the *Bacillus* species tested (Fig. 2A). Green gall extract showed higher activity in all cases, and indeed was the only extract effective against *S. aureus*, but the difference was significant only for *B. megaterium* and *S. aureus* (Fig. 2A). Of the gram-negative bacteria, gall extract antibacterial activity was demonstrated only for *Pseudomonas aeruginosa* with a small but significant difference between green and red gall extract (Fig. 2B).

Antifungal activity. Since no specific fungal pathogens of *S. wertheimae* or *Pistacia atlantica* are currently known, we tested fungi isolated from *Pistacia atlantica* leaves as well as representative fungi. Both green and red galls had a much higher content of essential oils than leaves (~1.5% v/w for green galls, ~1.2% v/w

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Figure 1. *Slavum wertheimae* galls on *Pistacia atlantica* tree. Notice the characteristic starting of reddening at the tips of the gall.

for red galls and $\sim 0.08\%$ v/w for leaves). Volatile constituents of the essential oils of galls delayed, although did not block, fungal growth in all tested fungal strains (Figs. 3 and 4). Volatile constituents of leaf essential oils, on the other hand, interfered only with growth of the *Penicillium* sp (Fig. 3B). Although no *Penicillium* growth was seen during the experiment (Fig. 3B), transferring the *Penicillium* disks (after 5 d) to a fresh PDA plate allowed its re-growth (data not shown).

Discussion

The humid and carbohydrate-rich interior cavities of the *S. wertheimae* galls are likely to provide favorable conditions for pathogen development. A given gall may support thousands of aphids for several months, during which they secrete large amounts of honeydew,²¹⁻²³ giving rise to high humidity and high sugar content that promote infestation by pathogens. Here we demonstrate that *S. wertheimae* galls have considerable antibacterial and antifungal properties distinct from those of intact leaves.

Interestingly, the antibacterial effectivity of gall extracts was especially notable against members of the *Bacillus* genus and against *Pseudomonas aeruginosa*, but not against another member of the *Pseudomonas* genus, *P. fluorescence*. Many members of the *Bacillus* genus are known insect pathogens,^{18,19} while *Pseudomonas aeruginosa* is a plant pathogen.¹⁸ *P. fluorescence*, on the other hand, is known to promote plant growth by controlling pathogenic organisms.²⁴ This suggests that the antibacterial activity is rather specific. On the fungi side, volatiles of gall essential oils had profound fungistatic effect against three filamentous fungi spp, two of which isolated from *Pistacia atlantica* leaves (Figs. 3 and 4). Although the exact *Pistacia atlantica* originated fungi species was not determined, they have been identified as belonging to the *Penicillium* genus, which is known to contain insect pathogenic species and is a common gall invader,¹⁰ and the *Aspergillus* genus, which is known to contain plant pathogenic species. The ecological and evolutionary relevancy of these findings needs further research.

It should be noted that previous studies have demonstrated antibacterial activity in organic extracts of *Pistacia* leaves.^{25,26} Although this seem to be of some contradiction to the results brought here (i.e., no detectable activity in the leaves), these studies used a much more coarse extraction methodology (e.g., very long organic solvent extracts) as opposed to our use of the more ecologically relevant water based extract.

The origin (i.e., endogenous metabolism of the galling insect vs. manipulation of host metabolism by the galling insect) was beyond the scope of the present study as was the identification of the antibacterial and antifungal compound(s). Nevertheless, aphid galls on *Pistacia* spp are known to accumulate large amounts of secondary metabolites compared with other plant parts, including mono-, sesqui- and triterpenes,^{14,16,27,28} tannins (unpublished data) and pathogenesis-related (PR) proteins.¹⁶ It is likely that a combination of these compounds provides the pronounced antimicrobial capacity of the galls.

Here we demonstrate that gall tissues have considerable negative effects on the growth of various bacterial and fungal pathogens. Our findings meet well with the “escape from enemy” hypothesis, which suggests that gall-inducing insects enjoy protection from various natural enemies,³ including microbial ones. These findings however, await deeper confirmation with natural *S. wertheimae* or *Pistacia* pathogens, once identified. Gall-formers are vulnerable to numerous predators (vertebrates and especially arthropods), parasitoids and pathogens (fungi and bacteria).^{1,4,5,7-11,29} The presence of a variety of defensive compounds in the galls of *Pistacia* can provide a protective solution against this wide range of natural enemies. From a broader view, given the considerable diversity of gall forming insects,² galls could greatly expand available sources for antimicrobial compounds for pharmaceutical and agricultural use.

Material and Methods

Host plants and gall-forming aphids. *Pistacia atlantica* (Anacardiaceae) is a deciduous dioecious tree of the eastern Mediterranean maquis and forests, and of the Irano-Turanian steppe. The tree serves as the exclusive host for the aphid *Slavum wertheimae* (Fordinae), which induces large cauliflower-shaped galls (Fig. 1). The galls are formed on the lateral buds in the spring, and several months later in the fall, just before the galls open, they turn red. Shortly afterwards, thousands of winged aphids migrate away from the open mature galls.³⁰ Because the gall’s color transformation might be associated with changes in the chemical composition of the galls, our bioassays were performed on both green and red galls.

Collection and preparation of plant material. In the fall (October 2009), we collected fully developed green galls, red galls and leaves, from five naturally growing *Pistacia atlantica* trees located near the campus of Oranim College in the Lower Galilee (Israel) (32°42' North, 35°7' East). Galls and leaves from all trees were pooled and frozen (at -20°C) pending analysis. Just before the beginning of the experiments, the preserved plant material was ground in liquid nitrogen to a fine powder, using mortar and pestle.

Bacterial strains. All bacterial strains tested in this study originated from the Oranim College culture collection. Since no specific bacterial pathogens of *S. wertheimae* or *Pistacia atlantica* are known, we tested the effect of galls and leaf powder aqueous extract on representative bacteria. The Gram positive strains used were *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus*. *Bacillus* strains were chosen for the study because many members of this genus are known to be insect pathogens.²⁰ The Gram negative strains used were *Pseudomonas aeruginosa* (a known plant pathogen),^{18,19} *Escherichia coli* B., *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Agrobacterium* sp and *Pseudomonas fluorescens*.

Fungal strains. Since no specific fungal pathogens of *S. wertheimae* or *Pistacia atlantica* are known, we tested the effect of essential oil hydrodistilled from galls and leaves on fungi isolated from *Pistacia atlantica* leaves, and on selected strains from the Oranim College culture collection. Three strains were tested: *Fomitopsis pinicola* (a common wood decay fungus), *Aspergillus* sp (a known plant pathogen), and *Penicillium* sp (some species are known insect pathogens). The two latter strains were isolated from *Pistacia atlantica* leaves.

Determination of antibacterial activities. Bactericidal activity was tested by a modification of the Kirby-Bauer disk-diffusion assay.^{31,32} Plant material powder (2 g wet weight) was mixed with 20 ml of 2% molten agarose (at 50°C) made with sterile water (giving 1:10 final w/v final concentration). The mixture was poured into a sterile empty Petri dish and allowed to solidify. As a control, the same mixture was prepared but plant powder was replaced by 2 g of distilled water. Tested bacteria were grown at 30°C with shaking (200 rpm), in liquid Lauria Bertoni (LB) broth to O.D.₆₀₀ ~ 0.6, spread on solidified LB agar medium and allowed to dry for about half an hour. Circles were collected from

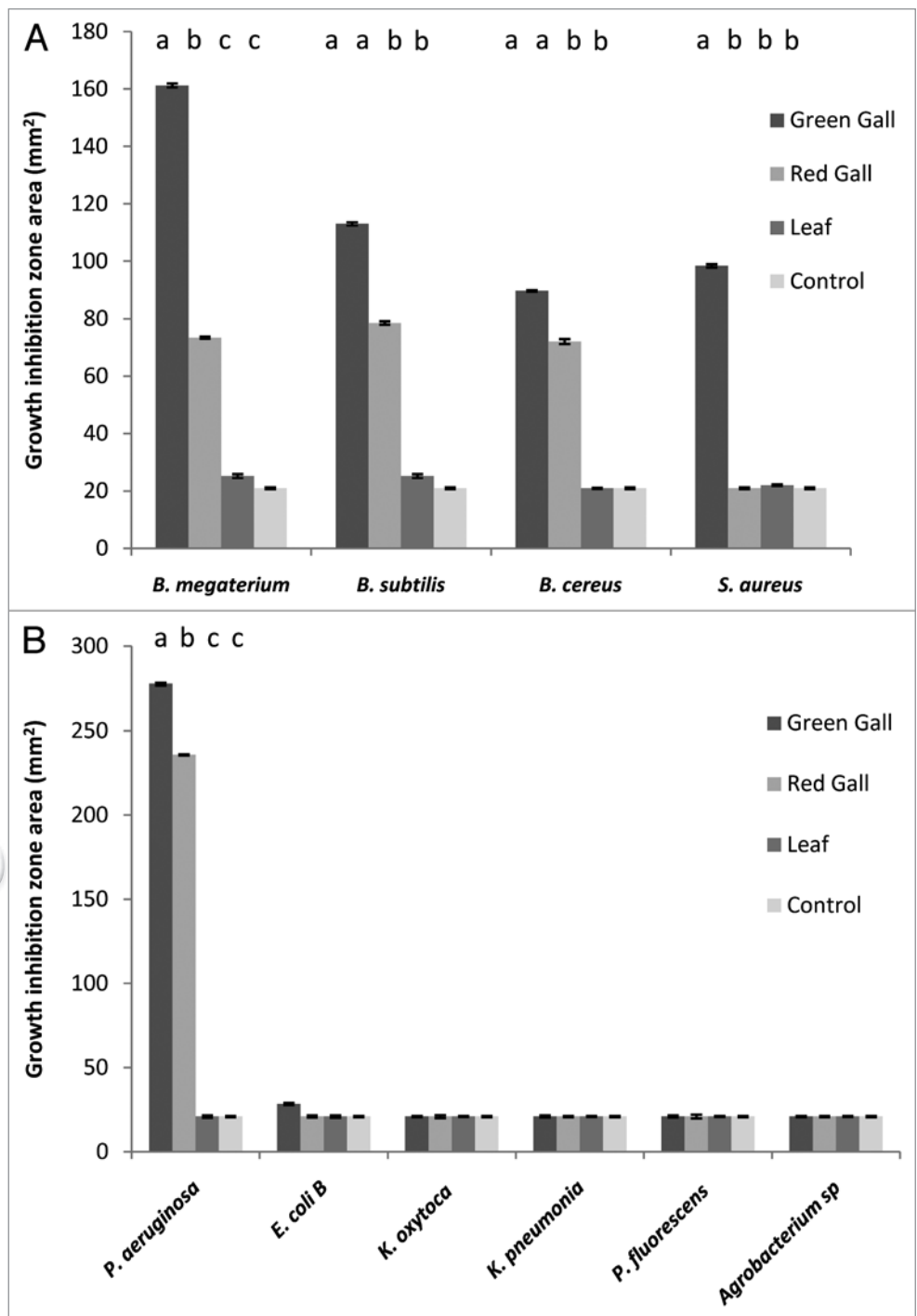


Figure 2. Average diameter of bacterial growth arrest halo formed around agarose disks containing green gall powder, red gall powder or leaf powder (4.9 mg plant material powder in each disk) and control (distilled water). (A) Gram-positive bacteria; (B) Gram-negative bacteria. In all cases disk diameter = 5 mm; n = 3 for green and red gall and for control; n = 6 for leaf. Error bars denote one standard error. Different letters above bars indicate significant differences (Bonferroni Multiple Comparison Tests, $p < 0.05$). One-way ANOVA indicated significant differences between the four treatments for five bacteria species (*B. megaterium* $F_{3,11} = 39.5$, $p < 0.001$; *B. subtilis*: $F_{3,11} = 23.7$, $p < 0.001$; *B. cereus*: $F_{3,11} = 48.4$, $p < 0.001$; *S. aureus* $F_{3,11} = 73.4$, $p < 0.001$; *Pseudomonas aeruginosa* $F_{3,11} = 188.6$, $p < 0.001$); whereas the differences in *E. coli* B, *K. oxytoca*, *K. pneumoniae*, *P. fluorescens* and *Agrobacterium* were not significant ($p > 0.05$ in all cases).

the solidified agar/plant powder mix using a 5 mm alcohol-sterilized cork borer, weighed and placed on the bacterial culture. Disk

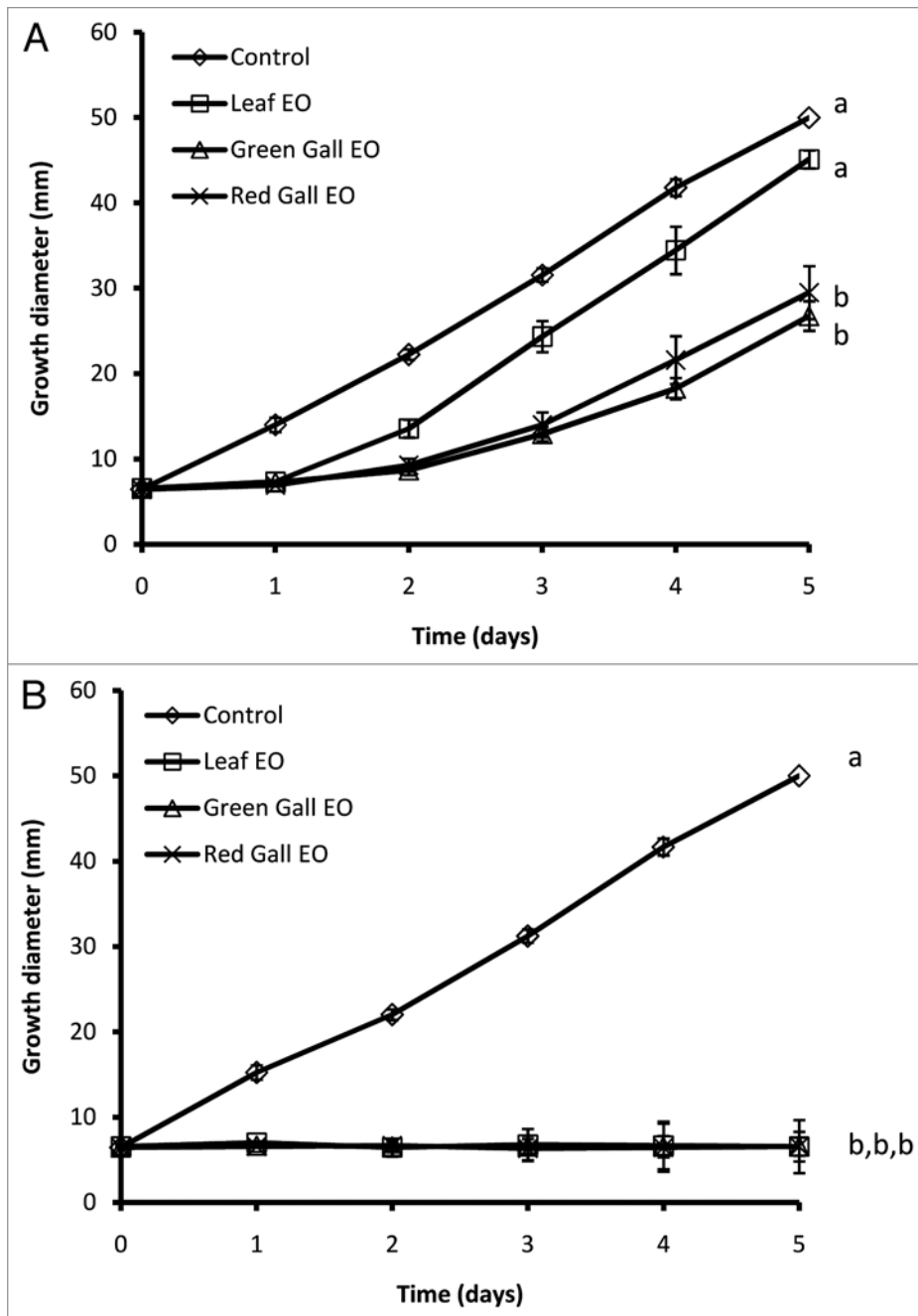


Figure 3. Effects of volatile constituents of essential oils volatiles from *Slavum wertheimae* galls or from leaves on growth of two fungus strains, (A) *Fomitopsis pinicola* and (B) *Penicillium* sp isolated from *Pistacia atlantica* leaves. N = 3 for each fungus-plant material combination; error bars denote one standard error; different letters indicate significant differences by day 5 (one-way ANOVA, followed by Bonferroni Multiple Comparison Tests; $p < 0.05$).

weight was 49 (± 5) mg. Cultures were allowed to grow for 24 to 48 h at 30°C, and growth inhibition was estimated by measuring three randomly selected diameters around the disk. All experiments were performed in triplicate, except for the experiments

with leaf powder, which were done in sextuplicate.

Determination of essential oil volatiles antifungal activity. Essential oils were extracted from the preserved and powdered galls and leaves by hydro-distillation. Green or red gall powders (50 g) were boiled in 250 ml distilled water and essential oils were collected in the side arm of a Clevenger apparatus. Leaf powder was treated similarly, but in order to obtain a sufficient quantity of essential oils we used 250 g leaf powder in one liter of water. Antifungal activity of the volatile constituents was tested as follows: fungi were seeded on potato/dextrose/agar (PDA) medium by placing agar circles (5 mm diameter) from the edge of a growing colony on a 5-cm-diameter PDA plate with the fungus facing the agar. The plates were incubated for 12 h at 30°C to ensure fungal viability, and then 15 μ l of essential oil from the leaf or the gall preparations was placed on the inside of the plate cover (final concentration 0.1% v/v). The plate was immediately sealed with six layers of parafilm and a layer of commercial insulation tape to prevent loss of essential oil vapor. Control plates were seeded as above but no essential oils were added. Plates were incubated at 30°C and fungal growth was estimated daily by measuring the advance of the mycelial front from the agar disk edge on three different radii (for the *F. pinicola*, *Fusarium* sp and *Penicillium* sp) or by coverage (for the *Aspergillus* sp). All experiments were performed in triplicate.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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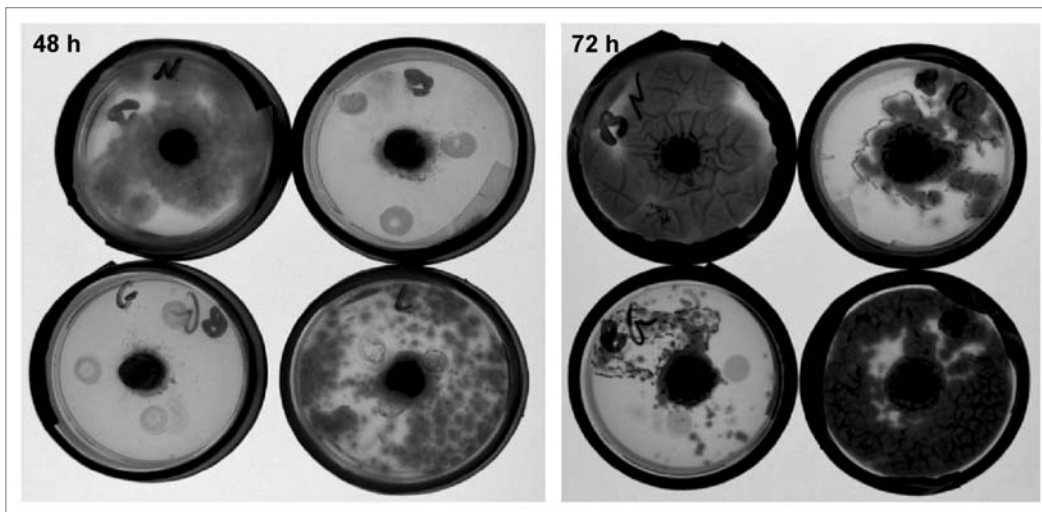


Figure 4. Representative Petri dish demonstrating the effects of volatiles constituents of essential oils from *Slavum wertheimae* galls and leaves on growth of *Aspergillus* sp isolated from *Pistacia atlantica* leaves. Left, after 48 h of growth; right, after 72 h of growth. For all time points—clockwise from top left corner: control, essential oils from red gall, essential oils from leaves, essential oils from green gall. The dark circle in the middle of each plate is the seeded fungus.

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