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Ability of (-)-Mesquitol Isolated from *Prosopis Juliflora* Heartwood to Inhibit Fungal and Bacterial Growth in a Laboratory Test

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Abstract

Studies were carried out to evaluate the ability of a novel flavonoid *(-)-mesquitol* isolated from *Prosopis juliflora* heartwood to inhibit fungal and bacterial growth. *Pycnoporus sanquineus (PS) and Gloephyleum trabeum (GT)* fungi were cultured in malt (30gm) and agar (40gm) inoculated with (none, 50, 100, 500 and 1000) ppm *(-)-mesquitol* in 9cm petri dishes and incubated in a sterile acclimatization chamber at 22°C and 70% RH. Fungal growth diameter was evaluated every 2 days for 14 days. Minimal Inhibitory concentration of *(-)-mesquitol* against bacterial strains: *Escherichia coli, Salmonella anterica, Staphylococcus aureus*, *Listeria monocytogenes* cultured in Trypcase Soja broth enriched with yeast extract and *Escherichia faecalis* cultured in Elliker broth was determined by two fold dilution of *(-)-mesquitol* in 96- well microtitre plates and incubated at 37°C. Bacterial growth was determined by measuring optical density of the suspension before and after 24 hrs incubation. Direct oxidation of *(-)-mesquitol* by laccase enzyme was carried out by measuring Ultra Violet (UV) absorbance at wavelength range (250nm- 650nm) every 2 minutes for 100minutes. *(-)-mesquitol* inhibited 70- 90% growth of *Pycnoporus sanquineus (PS) and Gloephyleum trabeum (GT)* fungi at 1000ppm respectively. Similarly it was able to inhibit over 22% of *Escherichia faecalis* bacteria at tested minimum concentration of 2.5mg/ml. In addition *(-)-mesquitol* induced laccase enzyme expression at UV absorbance range of 350 to 600nm similar to that of a known flavonoid (+)-catechin. These results suggest potential utilization of *(-)-mesquitol* and its derivatives in soap, cosmetic and shampoo industries.

Keywords

Flavonoid, (-)-Mesquitol, Prosopis Juliflora, Fungi

1. Introduction

Different plant species contain extractives such as tannins, flavanoids, lignans, stilbenes, terpenes and terpenoids that contribute to their resistance against fungal and bacterial bio-degraders [1, 2]. Of importance are the flavanoids classified into flavanones, flavones, chalcones, dihydroflavonols (flavanonols), flavonols, aurones, flavan-3-ols (catechins), flavan-3,4-diols (leucoanthocyanidins), anthocyanidins, isoflavonoïds, and neoflavonoïds [1, 3].

Indeed more than 4000 different types of flavonoids have been isolated from different plants [4]. The flavonoids constitute a large potential resource of natural antioxidants and radical scavengers for the food and pharmaceutical industries and for use as technical antioxidants [4]. Some flavonoids such as 3,4,7,8-tetrahydroxyflavanone and 4,7,8-trihydroxyflavanone have important antifungal, antibacterial and antitermitic properties with great potential for further development as biocides [5, 6]. Flavonoids such as naringenin and tamoxifen are reported to enhance the antibacterial, antiviral, or anti-cancer activities [7]. Some flavonoids such as taxifolin and quercetin show antifeedant activity against subterranean termites [8]. *Melicoccus bijugatus* fruits rich in catechins and other flavonoids are consumed for dietary and medicinal purposes, treatment of diabetes, cardiovascular and gastrointestinal disorders [9]. Tannins from Mimosa are useful as additives in glue for plywood bonding [10].

In our previous studies on plant extractives, we isolated

and extracted a novel flavonoid, (-)-mesquitol from the heartwood of *Prosopis juliflora*, without any noticeable impurities [11]. This study therefore aimed at understanding its ability to inhibit fungal and bacterial growth as a basis for its potential use in food and pharmaceutical industries.

2. Materials and Methods

2.1. Biological Organisms and Reagents

All fungal strains *Gloephyleum trabeum (GT)* and *Pycnoporus sanquineus (PS)*, brown and white rot fungi of significance respectively were obtained from CIRAD Forêt (Montpellier, France). The target bacterial strains: *Escherichia coli (strain CIP53126), Salmonella anterica (strain CIP81.32), Staphylococcus aureus (strain CIP4.83), Listeria monocytogenes (strain CIP82110)* were obtained from Nancy I University Medical School, France. All reagents and equipments unless otherwise stated were obtained from Sigma Aldrich Company.

2.2. Preparation of (-)-Mesquitol

(-)-mesquitol was prepared as described by [12] as follows: *Prosopis juliflora* heartwood was grounded to fine powder using a vibrating hammer mill, passed through a 115-mesh sieve and dried at 60°C to constant weights before extraction using acetone in accelerated solvent extractor (Dionex ASE 200). Extraction was performed in 33 mL cell size on 10gm of *P. juliflora* heartwood powder at 100°C under a pressure of 100 bars (3 static cycles of 5 minutes each). Three replicate extractions were done for each sample and the resulting extracts evaporated under vacuum to dryness and stored at -20°C awaiting its use.

2.3. Inhibition of Fungal Growth by (-)-Mesquitol

Growth inhibition of brown rot fungi *Gloephyleum trabeum (GT)* and white rot fungi *Pycnoporus sanquineus (PS)* by *(-)-mesquitol* was carried out as follows: Fungal mycelium was grown in 9 cm petri dishes filled with 20 ml of malt-agar medium (30 grams malt, 40 grams agar in 1L) treated to 50, 100, 500 and 1000 ppm of *(-)-mesquitol*. Control petri dishes were not treated with the *(-)-mesquitol*. Introduction of *(-)-mesquitol* was carried out after malt- agar medium sterilization (20 min, 120°C, 1 bar) by addition of the necessary quantity of *(-)-mesquitol* solubilised in 5ml of ethanol. Petri dishes were then inoculated in their centre with a 10 mm portion of healthy fungal colony and incubated in a sterile acclimatization chamber at 22°C and 70% RH.

Fungal growth was evaluated every 2 days by measuring the diameter of the colony estimated from the mean of two perpendicular diameters and expressed as a percentage of the room available for growth. Growth inhibition was calculated when the diameter of the control culture reached 9 cm according to the formula:

$$\text{Growth inhibition (\%)} = 100 \times (1 - d_1/d_0)$$

where d_0 is the diameter of the control culture and d_1 the diameter of the culture in the presence of *(-)-mesquitol*. All experiments were repeated two times.

2.4. Minimal Inhibitory Concentration of (-)-Mesquitol

The minimal inhibitory concentration (MIC) of (-)-mesquitol against bacteria was determined by the critical dilution method in 96- well microtitre plates. The target bacterial strains: *Escherichia coli (strain CIP53126), Salmonella anterica (strain CIP81.32), Staphylococcus aureus (strain CIP4.83), Listeria monocytogenes (strain CIP82110)* were cultured in Trypcase Soja broth enriched with yeast extract (TSB-YE broth) and *Escherichia faecalis (strain CIP76117)* cultured in Elliker broth to final optical density (OD_{620nm}) ≈ 0.01 by two fold dilution.

(-)-mesquitol was prepared to stock solutions of 10mg/ml and immediately frozen at -20°C. Under sterile conditions, test medium was prepared by two fold dilution of the 10mg/ml stock solution to the desired working solutions range (5mg/ml to 2.44×10^{-3}mg/ml) in the well plates. 100 µl of distilled water was introduced into each well of the 96-well microtitre plate followed by 20 µl bacterial culture (OD) ≈ 0.01. Positive controls were not exposed to *(-)-mesquitol*. All the plates were shacked (Titramax 100, Bioblock Fischer Scientific, Illkirch, France) for one minute before incubating at 37°C for 24 hours. The growth of bacteria was followed by measuring OD_{620nm} of bacterial suspension using Titertek multiscan MCC/340P, version 2.20 (Huntsville, Al) densitometer before and after 24 hours of incubation. Each test was triplicated. Inhibition of *(-)-mesquitol* on bacterial growth was evaluated as below:

$$\text{\% Inhibition} = (OD_c - OD_t) / OD_c \times 100$$

where OD_c is optical density control, OD_t is optical density in presence of *(-)-mesquitol* after 24 hours. MIC value (mgL^{-1}) is the inverse of the highest dilution where no growth is detected.

2.5. Oxidation of (-)-Mesquitol by Laccase Enzyme

Direct oxidation of *(-)-mesquitol* by laccase enzyme was carried out as follows: In a test tube, 5µl of 10mg/ml *(-)-mesquitol*, 485 µl of Phosphate buffer (pH 6.5, concentration 100Mm) and 10µl laccase enzyme at concentration of (5040µI/ml) were tested for UV absorbance (UV-spectrophotometer Cary 50 scan). Oxidation of *(-)-mesquitol* was followed by reading UV absorbance every 2 minutes for 2 hours at wavelength range of 250nm to 650nm.

3. Results and Discussions

3.1. Inhibition of Fungal Growth by (-)-Mesquitol

Figure 1 shows % growth inhibition of *P. sanguineus and G. Trabeum* fungi at increasing concentrations of *(-)-*

mesquitol and after 14 days.

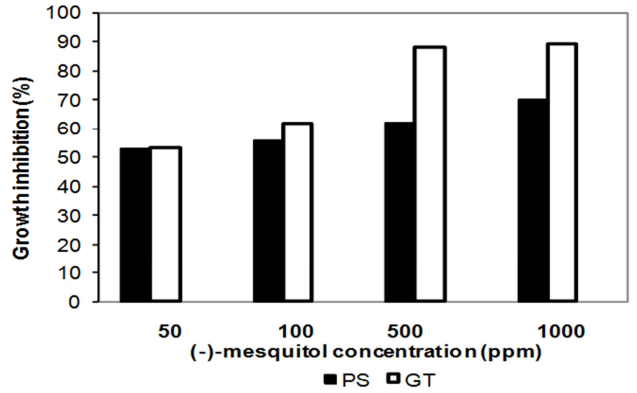

Figure 1. *Percentage (%) fungal growth inhibition at different concentrations of (-)-mesquitol.*

For the two fungi tested *P. sanguineus and G. Trabeum, %* fungal growth inhibition increased with increasing *(-)-mesquitol* concentration. Similarly growth of *P. sanguineus and G. Trabeum* under 50, 100, 500 and 1000ppm of *(-)-mesquitol* measured every 2 days for 14 days was inhibited in all the tested concentrations and at different times (figure 2).

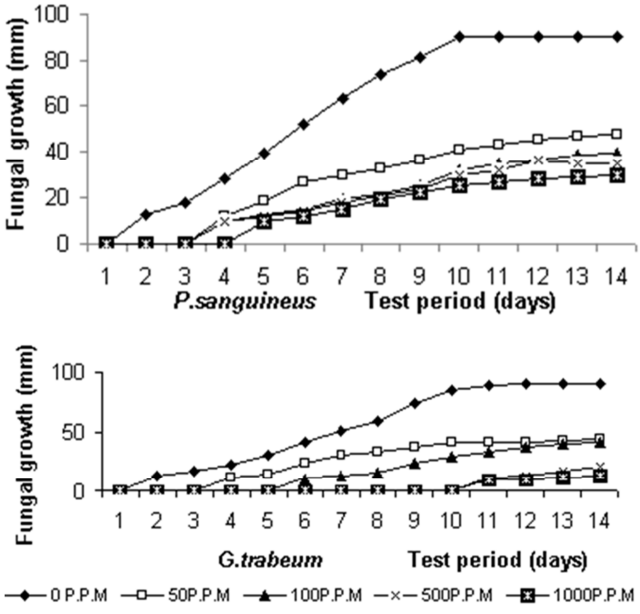

Figure 2. *Fungal growth inhibition by (-)-mesquitol at different test periods.*

This behavior is probably associated with detoxification of *(-)-mesquitol* by fungal enzymes, allowing further development of the fungus. However, their action seems to stem from a fungistatic rather than a fungicidal effect. Indeed, independently of the tested *(-)-mesquitol* concentration, development of the mycelium on treated medium started after a more or less lengthy inhibition period for the two fungi. During this period, fungal activity was detected by the formation of a colored area around the fungal inoculate. This behavior is probably associated with detoxification of the medium by fungal enzymes, allowing further development of the fungus. Increasing *(-)-mesquitol* concentration increased the inhibition period. According to these observations, it seems that *(-)-mesquitol* possess strong fungistatic properties even at low concentrations which increase with increasing concentration. It is therefore suggested that *(-)-mesquitol* like other flavonoids previously isolated from plants have fungicidal activity and excellent free radical scavengers (antioxidants) [13, 14, 15, 16]. Free radical scavenging activity of flavonoids is particularly important because both white-rot and brown-rot fungi are believed to use radicals to disrupt cell walls [17]. Brown rot fungi such as *Gloephyleum trabeum (GT)* are equipped with cellulase that break down the linear structure of wood and leaves the brown cubical remnants of lignin [18]. The optimal moisture content (MC) in wood for brown rot attack is 30-70%[19]. Oxalic acid ($C_2O_4H_2$), the strongest of the organic acids (PKa_1=1.23, PKa_2=4.26) is secreted by both brown and white rot decay fungi and has been connected to various aspects of the Fenton reaction in brown rot decay [20]. Oxalic acid secreted into the wood cell lumen quickly dissociates into hydrogen ions, decreases the pH of the medium and forms complexes with various cations. The reported tolerance of brown rot fungi to copper appears to be due to the complexing ability of oxalate with copper [20].

On the other hand, white rot fungi such as *Pycnoporus sanquineus* belongs to the class *Ascomycota* and *Basidomycota* [21]. *Ascomycota* is distinguished by sexual reproduction in so-called *ascus*. White rot fungi produce laccases and peroxidases that cleave the ring structures of lignin and celluloses hence break down the linear cellulose molecules. Indeed, laccases enable oxidation phenolic substrates and transformation of synthetic compounds to radicals that mediate attacks [22]. Our findings suggest that (-)-mesquitol a flavanoid has important antifungal properties. It seems therefore interesting to investigate if it is oxidized by the commercially available laccase enzyme in order to explain the observed fungistatic properties.

3.2. Effect of (-)-Mesquitol on Laccase Activity

It is reported that white rot fungi attack substrate by several enzymes such as cellulases, peroxidases or laccase, while the initial stages of brown-rot decay are caused by non enzymatic oxidative degradations involving hydroxyl radicals [23].

Figure 3 and 4 presents UV absorbance at wavelength range of 250nm to 650nm for the different assays of *(-)-mesquitol* and (+)-catechin as reference flavonoid before (TO) and after 100 minutes.

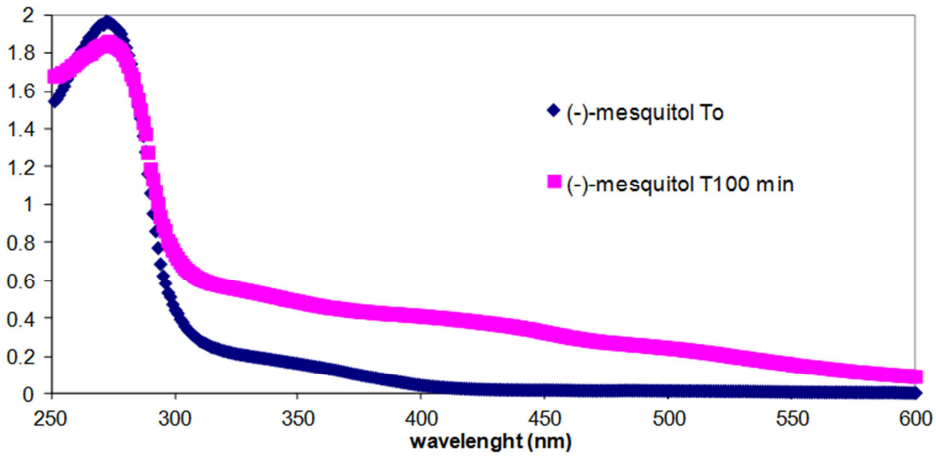

Figure 3. *UV absorbance at wavelength range of 250nm to 650nm for the different assays of (-)-mesquitol before and after 100 minutes.*

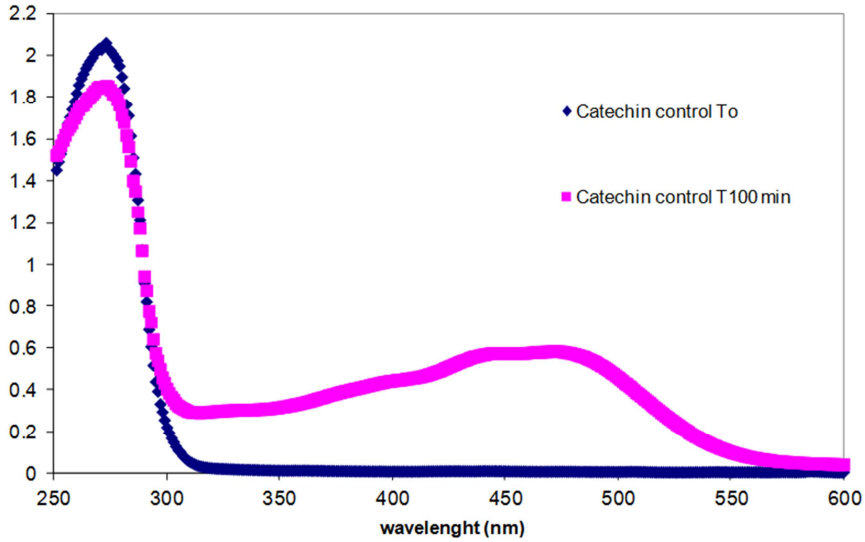

Figure 4. *UV absorbance at wavelength range of 250nm to 650nm for the different assays of (+)-catechin.*

Evolution of UV absorbance with time depends on the nature of the tested product. After 100 minutes, (+)-catechin is strongly oxidized by laccase as demonstrated by the large increase of the detected absorbance measured between 350 to 600 nm region. In the same time, UV spectra of (-)-mesquitol exhibit also an important increase of absorbance in the region between 350 to 550 nm. These results demonstrated that (-)-mesquitol is also oxidized by laccase. The two flavonoïds tested can be therefore considered as sacrificial antioxidants.

Indeed, laccase production has been correlated with fungal degradation in particular oxidation of catechin. (-)-mesquitol induce laccase expressions. Initial stages of fungal colonization correlate with (-)-mesquitol degradation, requiring laccase activity whereas the other degrading systems involving peroxidases and polysaccharide hydrolases are still repressed [24]. It is therefore probable to conclude that fungistatic properties observed previously associated with more or less important growth inhibition periods are explained by the ability of laccase enzymes to oxidize (-)-mesquitol.

3.3. Minimal Inhibitory Concentration of (-)-Mesquitol

Table 1 shows % bacterial growth inhibition of (-)-mesquitol (0.6 to 5.0 mg/ml) against the target bacterial strains: Escherichia coli, Salmonella anterica, Staphylococcus aureus, Listeria monocytogenes and Escherichia faecalis.

Table 1. *% bacterial growth inhibition by (-)-mesquitol.*

(-)-mesquitol (mg/ml)	5.0	2.5	1.3	0.6
Bacteria				
E. coli	-	-	-	-
S. anterica	-	-	-	-
S. aureus	-	-	-	-
E. faecalis	≥ 22	22	-	-
L. monocytogenes	-	-	-	-

(-)-mesquitol inhibited the activities of *E. faecalis* (MIC ≈ 2.5mg/ml) but was less important in other bacterial strains tested. Even if the results obtained showed poor antibacterial activity it is important to notice that concentrations used

during this study are relatively low. Indeed, a recent study aimed to evaluate antimicrobial activity of the isolated compounds from *Treculia africana* and *Treculia acuminata* [25] indicates MIC values for catechin of 2, 10 and 20 µg/ml for *E. coli*, *S. aureus* and *E. faecalis* respectively. MIC values of crude plant extracts are generally higher and comprised between 50 and 200 µg/ml according to their chemical composition. It is therefore necessary to perform additional experiments to evaluate the MIC of

(-)-mesquitol, which should be probably slightly higher than the maximal concentration tested in our study. Indeed bacteria of the class *Actinomycetes* are normally the first colonizers of substrates. They create conditions necessary for fungal attack by altering the nutrient status and production of synergistic secondary metabolites [26]. Bacterial attack thereafter follows by utilising resulting free water to propagate [27]. These results suggest utilization of *(-)-mesquitol* for the development of antifungal and antimicrobial drugs useful for the treatment of infections associated to microorganisms.

4. Conclusion

(-)-mesquitol isolated from *Prosopis juliflora* heartwood inhibit 70- 90% growth of *Pycnoporus sanquineus (PS) and Gloephyleum trabeum (GT)* fungi at 1000ppm respectively. Similarly it was able to inhibit over 22% of *Escherichia faecalis* bacteria at tested minimum concentration of 2.5mg/ml. In addition *(-)-mesquitol* induced laccase enzyme expression at UV range of 350 to 600nm similar to that of a known flavonoid (+)-catechin. These results suggest utilization of *(-)-mesquitol* and its derivatives in soap, cosmetic and shampoo industries.

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