# Antioxidant and antimicrobial activity of propolis extracts

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**Abstract.** This study aimed to compare the antioxidant and antimicrobial properties of four propolis extracts: two subcritical water extracts (WP1 and WP2 at 105°C and 135°C), a 10% propylene glycol extract (PGP, w/v, 70°C), and a 10% ethanol extract (ETOHP, w/v, 22°C). The novelty of this work lies in the direct comparative evaluation of these extraction methods under comparable experimental conditions and in assessing their impact on biological activity. Antioxidant capacity was determined using DPPH, ABTS, FRAP, and CUPRAC assays and interpreted in relation to total phenolic content (TPC). Significant differences ($p \leq 0.05$) were observed among the extracts. ETOHP exhibited the highest DPPH inhibition (75.73±0.76%) and TPC (37574.71±4451.55 mg GAE·$L^{-1}$), while PGP showed the highest FRAP (28973.67±1500.52 µmol TE·$L^{-1}$) and CUPRAC values (14945.53±138.59 µmol TE·$L^{-1}$). WP1 demonstrated the strongest ABTS scavenging activity (95.61±1.22%). Antimicrobial activity, evaluated by the agar well diffusion method, revealed that ethanol and propylene glycol extracts exhibited the strongest antibacterial effects, particularly against *Staphylococcus aureus* and *Listeria monocytogenes*. Antifungal activity was also observed, with *Penicillium chrysogenum* showing the highest sensitivity. These results indicate that propolis extracts possess significant bioactive potential for application in food systems, although further validation in real food matrices is required.

## 1 Introduction

Propolis is a natural resinous substance produced by honeybees (*Apis mellifera*) from plant exudates, which has attracted considerable scientific interest due to its complex chemical composition and diverse biological activities. It is rich in phenolic compounds, flavonoids, aromatic acids, and esters, which are primarily responsible for its pronounced antioxidant and antimicrobial properties [1,3]. Owing to these characteristics, propolis has been increasingly investigated as a natural alternative to synthetic additives in various industrial applications, particularly within the food sector [4].

In recent years, growing consumer demand for clean-label products and safer preservation strategies has intensified the search for natural bioactive compounds capable of extending shelf life and improving food quality [5]. In this context, propolis has emerged as a promising candidate due to its ability to inhibit the growth of spoilage and pathogenic microorganisms, as well as to reduce oxidative degradation processes [2,6]. These properties are particularly relevant for food systems that are highly susceptible to microbial contamination and lipid oxidation.

The biological activity of propolis is strongly influenced by its chemical composition, which in turn depends on several factors, including botanical origin, geographical location, and, importantly, the extraction method employed [1,7]. The efficiency of extraction plays a crucial role in determining the yield and profile of bioactive compounds. Conventional extraction techniques predominantly rely on ethanol as a solvent due to its high efficiency in solubilising phenolic constituents [8]. However, the presence of residual alcohol may limit its applicability in certain food systems and for specific consumer groups.

Consequently, increasing attention has been directed towards the development of alternative extraction approaches that are more suitable for food-related applications. These include the use of propylene glycol and water-based systems, as well as advanced techniques such as subcritical water extraction (SWE), which enables the recovery of bioactive compounds under controlled temperature and pressure conditions without the use of organic solvents [9,10]. Such methods are considered more environmentally sustainable and potentially more compatible with food applications.

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Although numerous studies have investigated the influence of extraction solvents and conditions on the chemical composition and biological activity of propolis [1,7,8], direct comparative analyses conducted under comparable experimental conditions remain relatively limited [10]. A systematic comparison of different extraction methods is essential for a more comprehensive understanding of the relationship between extraction technique, chemical composition, and resultant biological activity.

Due to their susceptibility to microbial spoilage and oxidative deterioration, meat and meat products represent one of the most relevant food systems in which natural bioactive compounds such as propolis may find practical application [6,11]. The incorporation of propolis-derived extracts into such systems could contribute to improved safety, extended shelf life, and reduced reliance on synthetic preservatives.

Therefore, the aim of the present study was to perform a comparative evaluation of propolis extracts obtained using different extraction methods, including subcritical water, propylene glycol, and ethanol, and to assess their antioxidant and antimicrobial activity under comparable experimental conditions.

## 2 Materials and Methods

### 2.1 Materials

#### *2.1.1 Propolis samples*

The propolis sample used in this study was collected from an apiary located near the town of Bansko, in the vicinity of the Krinets Dam (Southwestern Bulgaria) harvest in 2025. The sample was stored at ambient room temperature prior to extraction.

Before extraction, the propolis was frozen at –18°C and subsequently ground using a Philips HR2041/00 blender. This procedure ensured greater sample homogeneity and increased the surface area available for the efficient extraction of bioactive compounds.

#### *2.1.2 Extraction procedure*

Two water propolis extracts, labelled WP1 and WP2, were obtained by subcritical water extraction (SWE). The extraction was performed at two temperature conditions, 105 °C for WP1 and 135 °C for WP2. The extraction time was 5 min, and the solid-to-solvent ratio was approximately 1:24 (w/w). In both cases, the pressure conditions were controlled to maintain water in the liquid (subcritical) state throughout the extraction process, corresponding to typical subcritical water extraction conditions.All extraction experiments were conducted in triplicate.

The final concentrations of the extracts, expressed as total solids, were 1.7% for WP1 and 1.4% for WP2. The extraction procedure was adapted from Dobreva [8] which provides full technical details regarding the operating principles of SWE.

Subcritical water extraction (SWE) is a technique that employs water as a solvent at temperatures above its boiling point while maintaining sufficient pressure to keep it in the liquid state. Under these conditions, the dielectric constant of water decreases, enhancing its ability to dissolve less polar bioactive compounds, including phenolic acids and flavonoids. Consequently, SWE is considered a sustainable alternative to conventional organic solvents for the extraction of bioactive compounds from natural materials such as propolis.

The third extract (PGP) was prepared by extracting propolis at a concentration of 10% (w/v) in a 20% propylene glycol (analytical grade, ≥ 99% purity)/water solution (v/v). The extraction was carried out at 70°C for 1 hour with continuous stirring. After cooling, the extract was filtered and was stored in the dark at 4°C until further analysis.

The fourth extract, referred to as ETOHP, was obtained via ethanol extraction. A 10% (w/v) suspension of propolis in 70% (v/v) ethyl alcohol (analytical grade, 96% purity) was prepared and maintained in darkness at ambient temperature for 24 hours. Following extraction, the solution was filtered and stored in the dark at 4°C pending analysis.

The antioxidant capacity of each extract was assessed by various additional assays (DPPH, ABTS, FRAP and CUPRAC) and correlated with the total phenolic content.

The antibacterial and antifungal properties of the extracts were evaluated against a panel of food-related bacterial and fungal strains.

### 2.2 Antioxidant activity

#### *2.2.1 Radical scavenging capacity*

The 1, 1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) assay was performed according to the method of Brand – Williams [9], with modifications after Dinkova [10]. The absorbance of the decolourised solution was measured at $\lambda$ = 517 nm. In addition, the radical scavenging capacity against the 2, 2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) was determined at $\lambda$ = 734 nm, following the procedure described by Shopska [11]. The radical scavenging activities obtained from both assays were expressed as percentage inhibition (percentage inhibition capacity).

#### *2.2.2 Transition metal reducing activity*

The ferric ($Fe^{3+}$) reducing ability (FRAP assay) was determined at $\lambda$ = 593 nm according to the method of Benzie and Strain [12], with modifications after Dinkova [10]. In parallel, the cupric ($Cu^{2+}$) reducing antioxidant capacity (CUPRAC assay) was assessed at $\lambda$ = 450 nm following the procedure described in detail by Shopska [11]. All measurements were performed using a Helios Omega UV-Vis spectrophotometer equipped with VISIONlite software (Thermo Fisher Scientific, Madison, USA).

### 2.3 Determination of total phenolic content

The method is based on the reaction between phenolic compounds and the Folin–Ciocalteu reagent and the absorbance was measured at λ = 760 nm [13]. The standard curve was prepared using a gallic acid and results were presented as gallic acid equivalent. $L^{-1}$ (GAE. $L^{-1}$).

### 2.4 Antimicrobial activity of propolis extracts

The antimicrobial activity of the propolis extracts was determined using the agar well diffusion method. [14, 15].

The antibacterial activity of the extracts was evaluated against selected foodborne pathogenic bacteria, including *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Salmonella enterica* subsp. *enterica* ser. *Enteritidis* ATCC 13076, and *Listeria monocytogenes* ATCC 8787, as well as food spoilage fungi, including *Aspergillus niger* ATCC 9029, *Penicillium chrysogenum* ATCC 28089, *Fusarium verticillioides* ATCC 38932, and *Mucor* spp. The microbial strains were obtained from the American Type Culture Collection (ATCC, USA). These microorganisms were selected due to their relevance as common foodborne pathogens and spoilage organisms associated with the microbiological deterioration of food products.

Bacterial strains were cultivated on Luria–Bertani agar supplemented with glucose (LBG agar), with the following composition per litre: tryptone (10 g), yeast extract (5 g), NaCl (10 g), glucose (10 g), and agar (15 g), and incubated at 37 °C for 24 h under conditions suitable for the growth of mesophilic pathogenic bacteria. Fungal strains were cultured on Wort agar (HiMedia), a medium commonly used for the cultivation of fungus and yeasts in food microbiology, containing malt extract (30 g), peptone (5 g), and agar (15 g) per litre, and incubated at 30 °C for 3–7 days to allow adequate sporulation [14,15]. Microbial suspensions were prepared to match the McFarland 0.5 turbidity standard, corresponding to approximately $1.5 \times 10^8$ CFU $mL^{-1}$. Spore suspensions of the cultures were prepared after incubation on Wort agar and adjusted to concentrations of approximately $10^6$–$10^7$ CFU $mL^{-1}$.[14].

The prepared microbial suspensions were used to inoculate Petri dishes containing LBG agar or Wort agar (HiMedia). After solidification of the agar, wells with a diameter of 6 mm were aseptically created, and 60 µL of each propolis extract were dispensed into the wells. The inoculated plates were then incubated at 37 °C for bacterial cultures and at 30 °C for fungus. [14, 15].

Antimicrobial activity was assessed by measuring the diameter of the inhibition zones (mm) surrounding each well after incubation. Bacterial inhibition zones were measured after 24 h of incubation at 37 °C, whereas fungal inhibition zones were recorded after 48–72 h of incubation at 30 °C. [14].

Statistical analysis

The results were presented as mean values ± SD (standard deviation), with each measurement performed in triplicate (n = 3). A one-way analysis of variance (ANOVA) was applied to evaluate the statistical significance of differences among the propolis extracts, followed by Tukey's post hoc test ($p \leq 0.05$). Different superscript letters (a, b, c, d) indicate statistically significant differences at the 95% confidence level. Statistical analysis was conducted using SPSS software.

### 3.1 Antioxidant activity of propolis extracts

The antioxidant activity of the propolis extracts, as shown in Table 1, varied depending on the extraction method employed. The ethanol extract (ETOHP) demonstrated the highest DPPH radical inhibition (75.73%), confirming its efficiency in extracting phenolic antioxidant compounds. This observation is consistent with previous studies indicating that ethanol is highly effective in solubilising phenolic constituents and flavonoids responsible for antioxidant activity [4,5].

Among the water extracts, WP2 (8.32%) exhibited stronger DPPH scavenging activity than WP1 (4.56%). This difference may be attributed to the higher extraction temperature applied in WP2 (135°C), which likely enhanced the release of thermally stable and less polar phenolic compounds.

Table 1. Antioxidant activity expressed as radical scavenging capacity

| Method | WP1 1.7% | WP2 1.4% | PGP 10% | ETOH P 10% |
|---|---|---|---|---|
| **DPPH, % inhibition capacity** | $4.56^{d}$ ±0.02 | $8.32^{b}$ ±0.04 | $6.95^{c}$ ±0.06 | $75.73^{a}$ ±0.76 |
| **ABTS, % inhibition capacity** | $95.61^{a}$ ±1.22 | $83.96^{b}$ ±2.02 | $34.45^{c}$ ±0.24 | $35.62^{c}$ ±0.71 |

[a-d] Means in a row without a common superscript letter differ ($p \leq 0.05$).

The results are consistent with previous studies indicating that the antioxidant activity of propolis extracts is closely associated with their content of phenolic compounds and flavonoids [5]. Similarly, Nichitoi et al. [5] reported that different extraction procedures can significantly influence the yield of phenolic constituents and, consequently, the antioxidant activity of propolis.

Conversely, the ABTS results indicated that WP1 exhibited the highest radical scavenging capacity (95.61%), followed by WP2 (83.96%), while both PGP and ETOHP demonstrated considerably lower inhibition (approximately 35%). This suggests that the lower extraction temperature used for WP1 (105°C) promoted

the preservation of hydrophilic and thermolabile compounds associated with ABTS radical neutralisation.

Such behaviour may be explained by the temperature-dependent changes in the physicochemical properties of water during subcritical extraction, particularly the decrease in dielectric constant at higher temperatures, which favours the extraction of less polar compounds [8].

Subcritical water extraction (SWE) therefore produced extracts with distinct antioxidant characteristics: WP1 showed greater efficacy against ABTS radicals, whereas WP2 demonstrated enhanced reactivity in the DPPH assay, highlighting the tunable nature of SWE in relation to extraction temperature.

In both the FRAP and CUPRAC assays, the ethanol extract (ETOHP) demonstrated the highest reducing capacity (Table 2), reflecting its high concentration of redox-active phenolic constituents. The propylene glycol extract (PGP) displayed moderate antioxidant activity.

This finding is in agreement with previous studies reporting that glycol-based solvents can extract significant amounts of bioactive compounds while maintaining good antioxidant potential [7].

Among the water extracts, WP2 (135°C) exhibited higher FRAP and CUPRAC values than WP1 (105°C), indicating that the elevated extraction temperature enhanced the release of metal-reducing and chelating antioxidant compounds. These findings demonstrate that both solvent polarity and extraction temperature exert a significant influence on the antioxidant capacity of propolis.

In this regard, subcritical water extraction (SWE) represents a customisable and environmentally sustainable alternative to conventional solvent-based methodologies.

Table 2. Antioxidant activity of the propolis extracts determined by transition-metal ($Fe^{3+}/Cu^{2+}$) reducing capacity

| Method | WP1 1.7% | WP2 1.4% | PGP 10% | ETOH P 10% |
|---|---|---|---|---|
| **FRAP, µmol TE. $L^{-1}$** | $3628.87^{c} \pm 104.64$ | $4171.65^{c} \pm 429.34$ | $28973.67^{b} \pm 1500.52$ | $255294.3^{a} \pm 6621.68$ |
| **CUPRAC, µmol TE. $L^{-1}$** | $2239.19^{d} \pm 42.23$ | $7385.50^{c} \pm 80.81$ | $14945.53^{a} \pm 138.59$ | $11981.36^{b} \pm 42.30$ |

[a-d] Means in a row without a common superscript letter differ ($p \leq 0.05$).

The data presented in Table 3 show that the ethanol extract (ETOHP) possessed the highest total phenolic content (37574.71mg GAE. $L^{-1}$), confirming the strong efficiency of ethanol in solubilising phenolic constituents. The propylene glycol extract (PGP) displayed a moderate total phenolic content of 13593.20 mg GAE. $L^{-1}$, whereas both subcritical water extracts exhibited lower, yet still considerable, levels of phenolic compounds.

Table 3. Total phenolic content (TPC)

| Method | WP1 1.7% | WP2 1.4% | PGP 10% | ETOH P 10% |
|---|---|---|---|---|
| **TPC, mg GAE. $L^{-1}$** | $4573.80^{c} \pm 211.75$ | $6402.44^{bc} \pm 607.58$ | $13593.20^{b} \pm 703.83$ | $37574.71^{a} \pm 4451.55$ |

[a-c] Means in a row without a common superscript letter differ ($p \leq 0.05$).

Among the SWE extracts, WP2 (135°C) exhibited a higher total phenolic content (TPC) of 6402.44 mg GAE. $L^{-1}$ compared with WP1 (4573.80 mg GAE. $L^{-1}$), indicating that the elevated extraction temperature facilitated the release of bound phenolic compounds, despite the lower total solids content. The TPC results correlate with the enhanced antioxidant activity observed for WP2 in the DPPH, FRAP, and CUPRAC assays, confirming that SWE temperature directly influences both phenolic yield and antioxidant performance.

This relationship between total phenolic content and antioxidant activity has been widely reported in the literature [5].

Overall, the findings demonstrate that the antioxidant capacity of propolis was significantly affected by the extraction solvent and operational parameters. Ethanol extraction produced the highest total phenolic content and exhibited the strongest radical-scavenging and reducing abilities, reaffirming its effectiveness in isolating phenolic antioxidants. The propylene glycol extract showed moderate activity, whereas subcritical water extraction (SWE) generated bioactive extracts with distinct antioxidant profiles.

The lower SWE temperature (105°C) favoured the preservation of hydrophilic and thermolabile compounds contributing to ABTS activity, while the higher temperature (135°C) enhanced DPPH, FRAP, and CUPRAC responses, likely due to the release of more thermally stable and less polar phenolic. These results highlight the potential of SWE as an environmentally sustainable and tunable technique for obtaining antioxidant-rich propolis extracts.

### 3.2 Antimicrobial activity of propolis extracts against pathogens and fungi

The antimicrobial activity of the different propolis extracts was evaluated against pathogenic microorganisms associated with foodborne illnesses

(Table 4) and fungi responsible for food spoilage (Table 5).

The results obtained for the control samples demonstrated that the extraction solvents (water, 70% ethanol, and 20% propylene glycol) exhibited no antibacterial activity against the tested microorganisms, confirming their role as negative controls.

Overall, the antimicrobial activity of propolis extracts was found to be strain-dependent, which is consistent with previous reports attributing this effect to the presence of phenolic acids, flavonoids, and other bioactive compounds capable of disrupting microbial cell membranes and interfering with essential metabolic processes [3].

Among the tested extracts, the subcritical water extracts exhibited the lowest antimicrobial activity. The extract WP1 (1.7%) showed no inhibitory effect against any of the tested microorganisms, behaving similarly to the negative controls. In contrast, WP2 (1.4%) demonstrated limited antifungal activity, inhibiting the growth of *Aspergillus niger* ATCC 9029 and *Fusarium verticillioides* ATCC 38932, with inhibition zones of 9.00 ± 0.00 mm and 8.00 ± 0.00 mm, respectively.

The propylene glycol extract (PGP 10%) exhibited antibacterial activity against all tested bacterial strains. The observed inhibition zones were 15.00 ± 0.00 mm for *Escherichia coli* ATCC 8739, 17.50 ± 0.41 mm for *Staphylococcus aureus* ATCC 6538, 16.50 ± 0.41 mm for *Salmonella enteritidis* ATCC 13076, and 17.50 ± 1.22 mm for *Listeria monocytogenes* ATCC 8787. Gram-positive bacteria demonstrated slightly higher sensitivity compared to Gram-negative bacteria.

This behaviour is widely reported and is associated with structural differences in bacterial cell walls, particularly the presence of an outer membrane in Gram-negative bacteria, which limits the penetration of bioactive compounds [4].

Regarding antifungal activity, the PGP extract showed moderate inhibition against all tested fungi, with similar inhibition zones of approximately 10.00–10.50 mm for *Aspergillus niger*, *Penicillium chrysogenum*, *Fusarium verticillioides*, and *Mucor sp.*, indicating a generally higher resistance of fungi compared to bacterial strains.

Table 4. Antimicrobial activity of propolis extracts against bacteria

| | **Diameter zone of inhibition, mm** | | | |
|---|---|---|---|---|
| **Extracts** | ***Escherichia coli* ATCC 8739** | ***Staphylococcus aureus* ATCC 6538** | ***Salmonella enteritidis* ATCC13076** | ***Listeria monocytogenes* ATCC 8787** |
| **WP1 1.7%** | NA | NA | NA | NA |
| **WP2 1.4%** | NA | NA | NA | NA |
| **PGP 10%** | 15.00±0.00 | 17.50±0.41 | 16.50±0.41 | 17.50±1.22 |
| **ETOH P 10%** | 15.33±0.94 | 18.33±0.94 | 17.33±0.47 | 14.33±0.47 |
| **Control 1 (d. Water)** | NA | NA | NA | NA |
| **Control 2 (d. Water)** | NA | NA | NA | NA |
| **Control 3 (20% PG)** | NA | NA | NA | NA |
| **Control 4 (70% ETOH)** | 6.67±0.47 | 7.33±0.47 | 7.67±0.47 | 6.67±0.47 |

NA, not active

The ethanol extract (ETOHP 10%) also demonstrated pronounced antimicrobial activity. In terms of antibacterial effects, similar inhibition zones were observed for both Gram-positive and Gram-negative bacteria, namely 15.33 ± 0.94 mm for *E. coli*, 18.33 ± 0.94 mm for *S. aureus*, 17.33 ± 0.47 mm for *Salmonella enteritidis*, and 14.33 ± 0.47 mm for *L. monocytogenes*, indicating no clear distinction in sensitivity between the two groups.

In addition, the ethanol extract exhibited strong antifungal activity, with inhibition zones of 13.00 ± 0.00 mm for *Aspergillus niger*, 19.33 ± 0.47 mm for *Penicillium chrysogenum*, and 15.00 ± 0.00 mm for *Fusarium verticillioides*. Among the tested fungi, *Penicillium chrysogenum* showed the highest sensitivity to the ethanol extract. No inhibitory effect was observed against *Mucor sp.*

The overall results indicate that propolis extracts obtained using propylene glycol and ethanol exhibit the highest antimicrobial potential. These extracts may be considered promising candidates for application in food systems, including meat products; however, their effectiveness should be further validated in real food matrices.

The findings confirm the potential of propolis extracts as natural bioactive agents with significant antimicrobial properties. These results are in agreement with previous studies reporting the effectiveness of propolis in improving microbiological stability and extending the shelf life of food products, including meat and meat products [3]. However, their effectiveness in real food systems may be influenced by matrix complexity, including interactions with lipids, proteins, and water activity.

These findings highlight the potential of propolis extracts as natural bioactive agents for food preservation. However, it should be emphasised that the present results are based on in vitro assays, and their effectiveness in real food matrices requires further validation. Future studies should focus on evaluating their performance in model and real food systems, particularly meat products [3].

Table 5. Antimicrobial activity of propolis extracts against fungi

| | Diameter zone of inhibition, mm | | | |
|---|---|---|---|---|
| **Extracts** | ***Aspergillus niger* ATCC 9029** | ***Penicillium chrysogenum* ATCC 28089** | ***Fusarium verticillioides* ATCC 38932** | ***Mucor* sp.** |
| **WP1 1.7%** | NA | NA | NA | NA |
| **WP2 1.4%** | 9.00±0.00 | NA | 8.00±0.00 | NA |
| **PGP 10%** | 10.50±0.41 | 10.50±0.41 | 10.50±0.41 | 10.00±0.00 |
| **ETOH P 10%** | 13.00±0.00 | 19.33±0.47 | 15.00±0.00 | NA |
| **Control 1 (d. Water)** | NA | NA | NA | NA |
| **Control 2 (d. Water)** | NA | NA | NA | NA |
| **Control 3 (20% PG)** | NA | NA | NA | NA |
| **Control 4 (70% ETOH)** | 7.33±0.47 | 8.00±0.00 | 8.33±0.47 | NA |

NA, not active

## 4 Conclusions

The results of this study demonstrate that the antioxidant and antimicrobial properties of propolis extracts are strongly influenced by the extraction solvent and processing conditions. Ethanol extraction yielded the highest phenolic content and radical-scavenging activity, whereas subcritical water extraction represents an environmentally sustainable approach for obtaining thermally stable antioxidant compounds.

These findings indicate that propolis extracts constitute a promising source of bioactive compounds with antioxidant and antimicrobial properties. Such characteristics support their potential application as natural preservatives in food systems, including meat products. However, as the present results are based on in vitro assays, further studies in real food matrices are required to confirm their effectiveness under practical conditions.

Abbreviations

The following abbreviations were used in this manuscript:

SWE Subcritical water extraction
DPPH 2, 2-diphenyl-1-picrylhydrazyl radical
ABTS 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
FRAP Ferric-reducing antioxidant power
CUPRAC Cupric reducing antioxidant capacity
TPC Total phenolic compounds
ATCC – American Type Culture Collection

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