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Research Paper

# Antimicrobial potential and chemical composition of agro-industrial wastes

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## ABSTRACT

Agro-industrial wastes are rich in bioactive compounds. Its use as a source of natural antimicrobials may provide alternatives to the food industry as it enables the replacement of synthetic preservatives by natural compounds, as well as the disposal of by-products and reduced environmental impact. Therefore, this study assessed the antimicrobial potential and chemical composition of agro-industrial wastes against pathogenic microorganisms of importance in foods. Beet stalk, peanut peel, Pinot Noir grape marc, Petit Verdot grape seed and marc, red grapes fermentation lees and guava bagasse wastes showed antimicrobial activity against *Staphylococcus aureus* and *Listeria monocytogenes*. The minimum inhibitory concentrations ranged from 0.78 to 25mg/ml. Wastes with antimicrobial activity showed the highest total phenolic compounds among the wastes studied (37.3 to 400.2g GAE/kg). Analyses by GC-MS allowed the identification of caffeic, gallic, ferulic and ρ-coumaric acids, besides flavonoids quercetin, myricetin and epicatechin on wastes that exhibited antimicrobial activity. This study demonstrates that agro-industrial wastes from wine and food processing industries could be used as source of research on new antimicrobial compounds for use by food and beverage industry as natural preservatives.

**Keywords:** Agro-industrial wastes; Antimicrobial activity; Bioactive compounds.

## INTRODUCTION

The use of antimicrobials in food has become increasingly necessary as the global economy boosts the production and transportation of food worldwide; however, to ensure the supply of high-quality food, the use of preservatives is essential (Davidson and Branen, 2005). The potential application of natural antimicrobial compounds by the food industry is huge, and studies on the incorporation of antimicrobials in food and to maximize their biofunctionality have been conducted worldwide (Naidu, 2000).

Studies have shown the presence of bioactive compounds in different types of agro-industrial wastes, representing valuable potential application in industry. Their reuse would reduce environmental risks caused by disposal, besides providing a

source of profitability for populations living around industrial regions (Anastasiadi, et al., 2008). Different types of waste can be used as a source of raw material in researches for natural antimicrobials. Studies have found bioactive compounds with antimicrobial activity in grape seeds (Adámez, et al., 2011) and in their marcs (Katalinic, et al., 2010), pomegranate peels (Al-Zoreky, 2009), lemon peels (Mahmud, et al., 2009), green walnut husks (Oliveira, et al., 2008), among others.

In this study, wastes from food and beverage industries and from large fruit and vegetable distribution centers were evaluated for the presence of antimicrobial compounds with activity against microorganisms commonly associated with food toxico-infections such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli*. In addition, the chemical composition of wastes that exhibited the greatest antimicrobial potentials was determined.

## MATERIALS AND METHODS

***Agro-industrial wastes*:** Guava bagasse (*Psidium guajava*), Cabernet Sauvignon, Pinot Noir (*Vitis vinifera*) and Isabella grape marcs (*Vitis labrusca*) wastes were collected in several Brazilian industries in the first half of 2009. Petit Verdot and Verdejo grape marcs, Syrah and Verdejo grape stems, Petit Verdot grape seeds and red grapes fermentation lees (*Vitis vinifera*) and tomato bagasse (*Solanum lycopersicum*) wastes were collected in the second half of 2009. Vegetable wastes – kale (*Brassica oleracea*), beet (*Beta vulgaris*), broccoli (*Brassica oleracea*) and turnip stems (*Brassica rapa*), carrot (*Daucus carota*) and radish leaves (*Raphanus sativus*), pumpkin (*Cucurbita* sp.) and passion fruit hulls (*Passiflora edulis*) – were collected from gardens and fairs in the first half of 2009. In the same period, artichoke leaves (*Cynara cardunculus*) and peanut peels (*Arachis hypogaea*) were also collected in industries. With the exception of peanut peel, all wastes were freeze-dried for 5 days at 60-100μHg and at -50°C (Liotop® L101) and stored at -18°C until use.

***Extraction procedure***: The freeze-dried agro-industrial wastes were ground in mechanical mill (IKA® A11). For preparation of extracts, samples (1:8 w/v) were immersed in ethanol (40:60) and methanol (30:70) solutions and kept in rest under refrigeration for 96 hours. Every 24 hours, the extracts were filtered in qualitative filter paper 12.5μm (Qualy®) and the retained material was added to the respective extraction solvent. The solvents present in the filtrates were removed under low pressure at 45°C (rotary evaporator Tecnal®), and the resulting aqueous phase was freeze-dried (Liotop®L101). The freeze-dried extracts were stored under refrigeration until the time of analysis. For the tests, the extracts were dissolved in tryptic soy broth (TSB).

**Antimicrobial activity**

***Microorganisms tested*:** Antimicrobial activity was evaluated on Gram-positive (*Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC 7644) and Gram negative microorganisms (*Salmonella Enteritidis* ATCC 13076 and *Escherichia coli* ATCC 25922), from the collection of strains of the Laboratory of Hygiene and Dairy – "Luiz de Queiroz" School of Agriculture (ESALQ/USP).

***Agar diffusion method:*** The screening of the antimicrobial activity of extracts was performed using the agar diffusion technique according to the Clinical and Laboratory Standards Institute-CLSI (CLSI, 2009a) and Duarte et al. (2003), with modifications. 200μl of standardized inoculums ($1x10^8$ CFU/ml) of each organism were transferred to 200ml of tryptic soy broth (TSB) plus 0.7% of bacteriological agar at 45°C in order to obtain final bacterial population of $1.5\ x10^5$ CFU/ml. With the aid of a sterile

graduated test tube, 70ml of inoculated agar were transferred to Petri dishes (150mm), in which wells of 8mm in diameter were produced by vacuum pump, within which 40µl of extracts were distributed (100mg/ml). Petri dishes were kept at rest for 1 hour at room temperature to allow the diffusion of extracts into the medium. As negative control, 40µl of TSB were used, and as positive control, 40µl of chlorhexidine solution 0.12% (v/v) were used. Triplicates were made for each extract tested.

***Minimum bactericidal and inhibitory concentrations (MIC/MBC):*** For MIC determination, the macrobroth dilution method was used, as described by the CLSI (CLSI, 2009b) in 96-well microplate. The concentrations of extracts were obtained by 2-fold serial dilution in the microplate, resulting in concentrations ranging from 25mg/ml to 0.78mg/ml after the addition of inoculated TSB (1-2x$10^5$ CFU/ml). The final volume for each well was 200µl. The controls were composed as follows: positive control (200µl of TSB added of 0.12% chlorhexidine v/v) and negative control (200µl of sterile TSB). Two hundred microliters of sterile TSB were used for broth sterility control. After incubation at 35°C for 24 hours, all wells received 30µl of resazurin (0.01% w/v) with the objective of verifying, through visual reading, in which wells bacterial growth was detected. Any evidence of color change was considered as indicative of bacterial growth. For the MBC determination, 10µl of broth were removed from the wells considered inhibitory and sown in Petri dishes containing tryptic soy agar (TSA), which were incubated at 35°C for 24 hours. The MBC was considered as the lowest concentration at which no growth of colonies on the surface of the culture medium was observed (Cabral, et al., 2009). The experiments were conducted in triplicate for each extract.

***Growth curves:*** The effect of extracts on the growth of microorganisms was assessed using 96-well microplates (Gutierrez, et al., 2008). All wells received 100µl of sterile TSB. The first well of each column was added of 100µl of extracts to be tested and then 2-fold serial dilution was performed with the aid of a multichannel micropipette, resulting in final concentrations ranging from 25mg/ml to 0.78mg/ml, after adding 100µl of inoculated broth (1-2x$10^5$ CFU/ml). Controls consisted of 200µl of inoculated TSB (negative control), 200µl of inoculated TSB added of chlorhexidine 0.12% v/v (positive control) and 200 µl of TSB, plus extract, without inoculum (white). The microplates were incubated in spectrophotometer (Victor™X3, PerkinElmer®) at 35°C for 18 hours and the absorbance readings were performed at intervals of 1 hour at 600 nm. Triplicates were made for each extract tested.

**Chemical composition**

***Determination of total phenolic compounds:*** The total phenolic content was determined with Folin-Ciocalteau reagent according to methodology described by Singleton et al. (1999). 500µl of extracts (10mg/ml) were mixed with 2.5ml of Folin-Ciocalteau reagent (1:10) and 2ml of sodium carbonate solution ($Na_2CO_3$) (4% w/v). After incubation in the dark at room temperature for 2 hours, the absorbance reading was performed at 740 nm in visible light spectrophotometer (Femto® Plus). The total phenolic content was expressed as gallic acid equivalent (GAE) in g per kg of sample (g GAE/kg) from the gallic acid standard curve. For the gallic acid, the curve was established by plotting concentration (µg/ml) *versus* absorbance (nm) ($y = 42.71x + 0.3187$; $R^2 = 0.9997$, where $y$ is the absorbance and $x$ is the concentration).

***Gas chromatography with mass spectrometry (GC-MS):*** The extracts that showed the best antimicrobial activities were submitted to gas chromatography with mass spectrometry (GC-MS) in order to determine their chemical composition according to

Proestos et al. (2006) and Markham et al. (1996). *Chromatographic analysis*: the extracts were analyzed by Shimadzu® gas chromatograph (Model GC-2010) coupled to a Shimadzu® mass spectrometer (QP 2010 Plus). The separation occurred in capillary column RTX5MS (30m x 0.25mm x 0.25μm). The injector temperature was 280°C and the injection volume was 0.5 μl in "splitless" mode. The interface was maintained at 280°C and the detector operated in the "scanning" mode (m/z 40-800). Chromatographic conditions were: initial temperature of 80ºC (1 min) heating to 250°C, at a rate of 20°C/min (1 min), heating to 300°C (5 minutes) at a rate of 6°C/min, heating to 310°C (10 minutes) at a rate of 15°C/min, and heating to 320ºC (10 minutes) at a rate of 20°C/min, totaling 40 minutes of analysis. The integration was done using the LabSolutions-CGMS software. Flavonoids, phenolic acids and derivatives were identified by comparison with data obtained from GC-MS, such as retention time and ionic fragmentation of authentic standards silanized and eluted under the same conditions, and with the Wiley 8 library.

***Statistical analysis*:** The statistical analysis was performed using the Statistical Analysis System software (SAS 2002). The Tukey's test (0.5% probability) was used to compare means.

## RESULTS AND DISCUSSION

***Antimicrobial activity:*** Preliminarily, the antimicrobial potential of extracts was qualitatively evaluated by agar diffusion method. Of the 20 wastes analyzed, 7 showed inhibition zones for at least one of microorganisms tested, namely: beet stems, peanut peels, Petit Verdot and Pinot Noir grape marcs, Petit Verdot grape seeds, red grapes fermentation lees and guava bagasse (Table 1). The quantitative analysis of the antimicrobial potential of extracts was performed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC); the extracts showing the lowest MIC against *S. aureus* and *L. monocytogenes* were, respectively, methanol extract of peanut peels (0.78mg/ml) and ethanol extract of guava bagasse (1.56mg/ml) (Table 1). The minimum bactericidal concentration of peanut peel extract was relatively low (1.56mg/ml). Studies have already shown the antioxidant activity of this waste (Davis, et al., 2010). However, there are no references as for its antimicrobial activity against Gram-positive and Gram-negative bacteria. Guava bagasse extract showed the highest antimicrobial activity against *L. monocytogenes*, although its bactericidal potential is reduced with a high MBC value (12.5mg/ml). The antimicrobial potential of guava parts such as leaves and bark has been investigated (Gutiérrez, et al., 2008); however, little is known about the reuse and utilization of wastes from guava processing by agro-industries.

The inhibitory activity of extracts of grape parts such as seeds against Gram-positive bacteria is in agreement with results found in previous studies (Adámez, et al., 2011). The antilisterial activity of grapes and winery byproducts – such as marcs, seeds and stems – enhances the potential for extraction of antimicrobial compounds in this type of waste (Anastasiadi, et al., 2008), although this study did not show this activity in stems of white grapes. Despite being a still unexplored winery byproduct, red grapes fermentation lees had one of the lowest MIC detected for *S. aureus* among the investigated wastes, as well as methanol extract of beet stems, and some studies have reported the antioxidant and antimicrobial potential of beet; however, using the tuber, not the stalks (Rey, et al., 2005).

None of the extracts inhibited the growth of *S.* Enteritidis and *E. coli*. Gram-negative bacteria have a second system of lipid bilayers, the outer membrane, which

hinders the penetration of antimicrobial substances, giving them a high resistance level (Schved, et al., 1994). These microorganisms commonly present minimum inhibitory concentration values significantly higher for many antibacterial agents when compared with Gram-positive bacteria (Höltje, 2004).

The effect of wastes on microbial growth was analyzed by the growth curves of *S. aureus* and *L. monocytogenes* in relation to the six most active extracts. For this, a comparison between two concentrations was made: MIC and concentration immediately below MIC, called sub-MIC. The analysis of growth curves confirmed the antimicrobial activity of extracts and their respective MIC. For all extracts evaluated, although not inhibiting the *in vitro* bacterial growth, the sub-MIC increased the lag phase when compared with their negative control curves (Figures 1 and 2). For *S. aureus*, the guava bagasse extract in its sub-MIC quadrupled the lag phase of bacterial growth, demonstrating its potential use even at concentrations lower than those found for MIC. For *L. monocytogenes*, the increased adaptation period of the microorganisms to the medium could also be observed, especially for the sub-MIC of the methanol extract of beet stalks (Figure 2).

***Chemical composition:*** The total phenolic compounds found for ethanol and methanol extracts of wastes ranged from 7.0 to 400.2g GAE/kg (Table 2). The extracts that showed antimicrobial activity against *S. aureus* and *L. monocytogenes* are among those with the highest total phenolic values, suggesting a correlation between antimicrobial activity and the presence of phenolic compounds. Extracts of peanut peel, Petit Verdot seeds and marc and red grapes fermentation lees had the lowest MIC and the highest total phenolic content values.

The chemical composition of extracts analyzed by GC-MS technique is presented in Table 3. Azelaic acid, a saturated chain dicarboxylic acid widely used in the treatment of acne and of recognized antimicrobial activity, is among the major components of the beet stalk extract (Gollnick and Schramm, 1998). Dicarboxylic acids such as succinic and azelaic acids, epicatechin, caffeic acid and ρ-coumaric acid were found in peanut peel extract and in the extracts of many other vegetables, with proven antimicrobial and antifungal activities (El-Massry, et al., 2009). A recent study has shown the presence of caffeic and syringic acids in grape marcs with antilisterial activity, which is in agreement with results of this study (Anastasiadi, et al., 2008). The previous authors also found large amounts of epicatechin in grape berries, marcs and seeds. In this study, Petit Verdot grape seed and marc extracts were the most abundant in epicatechin. Fermentation lees showed the presence of gallic and ferulic acids, and flavonoids myricetin and quercetin, compounds of known antimicrobial activity (Naidu, 2000). Epicatechin, quercetin and caffeic acid were the most abundant phenolic compounds in the guava bagasse extract, which are compounds with antibacterial activity commonly found in fruits and leaves (Gutiérrez, et al., 2008). Petit Verdot grape seed extracts showed predominance of epicatechin and presence of caffeic and gallic acids, which is in agreement with results found in previous studies on grape seeds (Anastasiadi, et al., 2008; Maier, et al., 2009).

## CONCLUSIONS

Agro-industrial wastes beet stalks, peanut peel, Petit Verdot grape seeds and marcs, red grapes fermentation lees and guava bagasse showed compounds with antimicrobial activity against *S. aureus* and *L. monocytogenes*, which are important bacterial pathogens in humans. The use of such wastes by the food industry becomes

viable, since it is a natural alternative to synthetic preservatives and avoids waste disposal into the environment, bringing benefits to both industry and consumers.

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**Table-1: Inhibition Zone (IZ) (mm), Minimum Inhibitory and Bactericidal Concentrations (MIC/MBC) (mg/ml) of ethanol and methanol extracts of agro-industrial wastes with antimicrobial activity.**

| Wastes | *S. aureus* | | | | | |
|---|---|---|---|---|---|---|
| | MeOH | | | EtOH | | |
| | IZ | MIC | MBC | IZ | MIC | MBC |
| Beet stem | 10.00±0.00 | 3.13 | 3.13 | - | - | - |
| Peanut peels | 18.00±0.00 | 0.78 | 1.56 | 20.00±0.00 | 0.78 | 1.56 |
| P. Noir grape marc | 10.67±0.57 | 6.25 | 6.25 | 10.00±0.00 | 6.25 | 6.25 |
| P. Verdot grape marc | 12.00±0.00 | 3.13 | 6.25 | 10.00±0.00 | 6.25 | 12.5 |
| Fermentation lees | 10.00±0.00 | 3.13 | 6.25 | 10.00±0.00 | 1.56 | 12.5 |
| Guava bagasse | 10.00±0.00 | 3.13 | 3.13 | 10.00±0.00 | 3.13 | 6.25 |
| P. Verdot seeds | 16.00±0.00 | 3.13 | 3.13 | 14.00±0.00 | 1.56 | 3.13 |
| | *L. monocytogenes* | | | | | |
| | MeOH | | | EtOH | | |
| | IZ | MIC | MBC | IZ | MIC | MBC |
| Beet stem | 12.00±0.00 | 12.5 | 25.00 | - | - | - |
| Peanut peels | 12.33±0.57 | 3.13 | 3.13 | 14.00±0.00 | 3.13 | 3.13 |
| P. Noir grape marc | 10.00±0.00 | 12.5 | 12.5 | 10.00±0.00 | 12.5 | 12.5 |
| P. Verdot grape marc | 10.00±0.00 | 12.5 | 25.00 | - | - | - |
| Fermentation lees | 10.00±0.00 | 6.25 | 25.00 | 10.00±0.00 | 12.5 | 25.00 |
| Guava bagasse | 12.00±0.00 | 3.13 | 12.5 | 12.67±0.57 | 1.56 | 12.5 |
| P. Verdot seeds | 12.00±0.00 | 6.25 | 12.5 | 10.00±0.00 | 6.25 | 12.5 |

- Averages of triplicates ± standard deviation
- MeOH: methanol extracts / EtOH: ethanol extracts
- -: No inhibition

**Table- 2: Total phenolic compounds (GAE/kg sample) of extracts from different agro-industrial wastes.**

| Wastes | Extracts | |
|---|---|---|
| | MeOH | EtOH |
| Peanut peels | 400.2 ± 6.1 $^{aA}$ | 374.5 ± 9.7 $^{aB}$ |
| Petit Verdot seeds | 348.0 ± 8.6 $^{bA}$ | 297.9 ± 12.6 $^{bB}$ |
| Petit Verdot grape marc | 186.1 ± 5.3 $^{cA}$ | 192.5 ± 3.9 $^{dA}$ |
| Pinot Noir grape marc | 161.9 ± 2.5 $^{dB}$ | 229.2 ± 1.6 $^{cA}$ |
| Fermentation lees | 124.2 ± 1.4 $^{eB}$ | 165.1 ± 2.7 $^{eA}$ |
| Verdejo grape stalk | 81.7 ± 0.5 $^{fA}$ | 81.6 ± 1.7 $^{fA}$ |
| Izabella grape marc | 64.8 ± 1.5 $^{gA}$ | 64.8 ± 1.5 $^{gA}$ |
| Beet stalk | 49.5 ± 5.7 $^{hA}$ | 51.0 ± 1.6 $^{hA}$ |
| Syrah grape stalk | 52.5 ± 1.3 $^{hB}$ | 70.7 ± 1.8 $^{fgA}$ |
| Guava bagasse | 37.3 ± 0.4 $^{iB}$ | 43.1 ± 0.9 $^{hiA}$ |
| Radish leaves | 35.3 ± 0.7 $^{iB}$ | 36.7 ± 0.3 $^{ijA}$ |
| Turnip stem | 31.6 ± 0.2 $^{ijA}$ | 30.4 ± 0.9 $^{jkA}$ |
| Kale stem | 24.9 ± 1.1 $^{jkA}$ | 23.6 ± 0.7 $^{klmnA}$ |
| Verdejo grape marc | 24.8 ± 0.2 $^{jkB}$ | 28.9 ± 0.4 $^{jklA}$ |
| Tomato bagasse | 22.1 ± 0.4 $^{jklB}$ | 28.1 ± 0.9 $^{jklA}$ |
| Passion fruit hull | 19.5 ± 0.9 $^{klB}$ | 24.2 ± 0.6 $^{klmA}$ |
| Broccoli stem | 18.0 ± 0.2 $^{klmA}$ | 17.3 ± 0.7 $^{lmnoA}$ |
| Carrot leaves | 13.5 ± 0.6 $^{lmnA}$ | 12.4 ± 0.4 $^{mnoA}$ |
| Artichoke | 9.4 ± 0.2 $^{mnB}$ | 11.9 ± 0.2 $^{noA}$ |
| Pumpkin hulls | 7.8 ± 0.6 $^{nA}$ | 7.0 ± 0.3 $^{oA}$ |

- Averages in rows (n=3) followed by different small letters show statistical difference at 5% (Tukey). Averages in columns (n=3) followed by different capital letters show statistical difference at 5% (Tukey)

**Table-3: Chemical composition of extracts of agro-industrial wastes with antimicrobial activity against pathogenic microorganisms.**

| Compounds [c] | RT | Percentage of relative area [a] | | | | | | Ion (m/z, abundance between parenthesis) |
|---|---|---|---|---|---|---|---|---|
| | | Agro-industrial wastes [b] | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | |
| Succinic acid | 6.19 | - | 0.63 | - | - | - | - | 147 (100), 73 (92) 44 (32), 55 (28); 247 ($M^+$) |
| Azelaic acid | 9.03 | 2.88 | 4.35 | 0.54 | 2.22 | 2.41 | 0.84 | 73 (100), 55 (40), 201 (30), 43 (30), 129 (26), 149 (26); 317 ($M^+$) |
| Syringic acid | 9.63 | - | - | 0.54 | 3.03 | - | - | 327 (100), 73 (69), 312 (62), 253 (33), 283 (19), 355 (6); 342 ($M^+$) |
| ρ-coumaric acid | 9.85 | - | 3.72 | - | - | - | - | 73 (100), 293 (96), 308 (73), 219 (64), 249 (56), 44 (21); 308 ($M^+$) |
| Gallic acid | 9.93 | - | - | 1.68 | 2.18 | - | 2.62 | 281 (100), 73 (89), 443 (23), 282 (22), 443 (23), 45 (12); 458 ($M^+$) |
| Ferulic acid | 10.35 | - | - | - | 2.04 | - | - | 73 (100), 117 (74), 313 (64), 132 (38), 43 (28), 55 (21); 338 ($M^+$) |
| Caffeic acid | 10.99 | - | 0.46 | 1.05 | 0.48 | 1.11 | 0.35 | 219 (100), 73 (90.38), 381 (20), 45 (15), 191 (12), 249 (10), 396 ($M^+$) |
| Epicatechin | 17.27 | - | 3.95 | 79.95 | 24.98 | 7.92 | 75.33 | 368 (100), 73 (61), 267 (9), 179 (8), 383 (7); 650 ($M^+$) |
| Quercetin | 20.68 | - | - | - | 3.13 | 1.42 | - | 647 (100), 73 (43), 207 (20), 559 (11), 281 (10), 575 (7),; 662 ($M^+$) |
| Myricetin | 21.25 | - | - | - | 1.70 | - | - | 73 (34), 207 (18), 647 (13), 281 (8), 217 (7), 307 (6); 735 ($M^+$) |

- [a] peak area in relation to total percentage of peak areas
- [b] 1. Beet stalks (methanol), 2. Peanut peel (methanol), 3. Petit Verdot grape marc (methanol), 4. Fermentation lees (ethanol), 5. Guava bagasse (ethanol), 6. Petit Verdot seeds (ethanol)
- [c] All compounds identified showed similarity percentage > 80%
- RT: retention time (min)

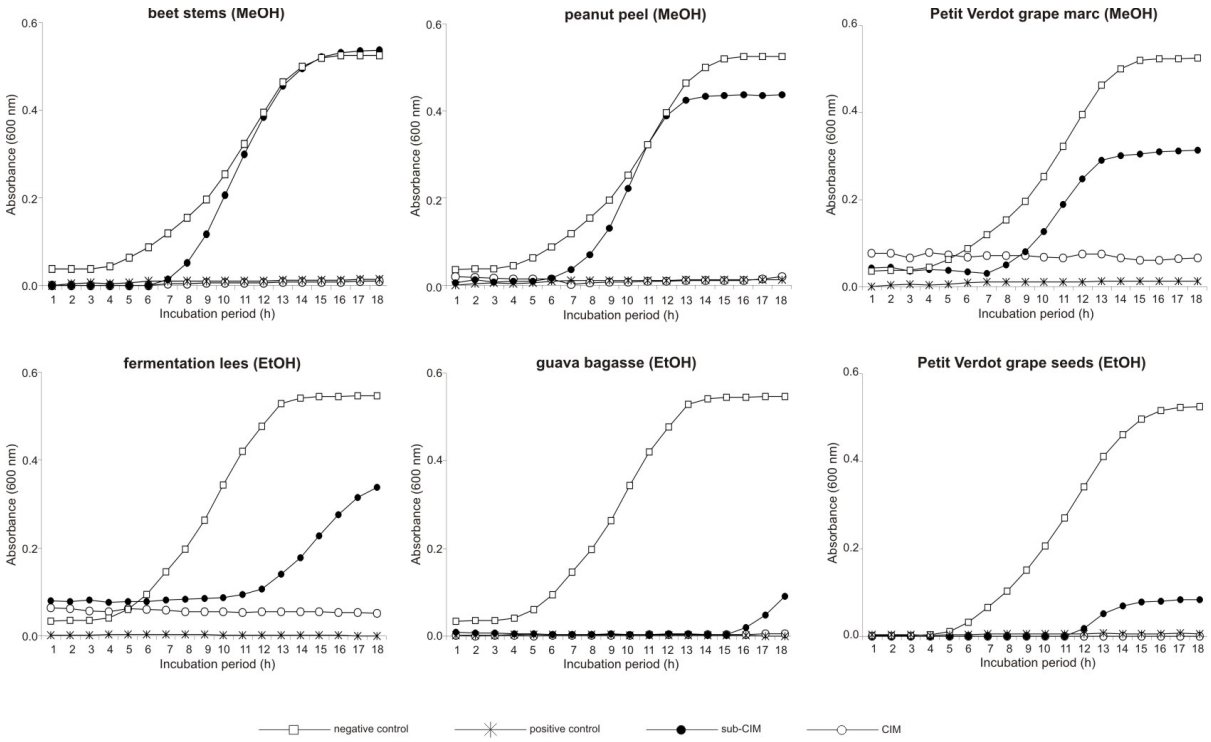


**Figure-1: Growth curves of *S. aureus* in relation to methanol (MeOH) and ethanol (EtOH) extracts of agro-industrial wastes at different concentrations.**

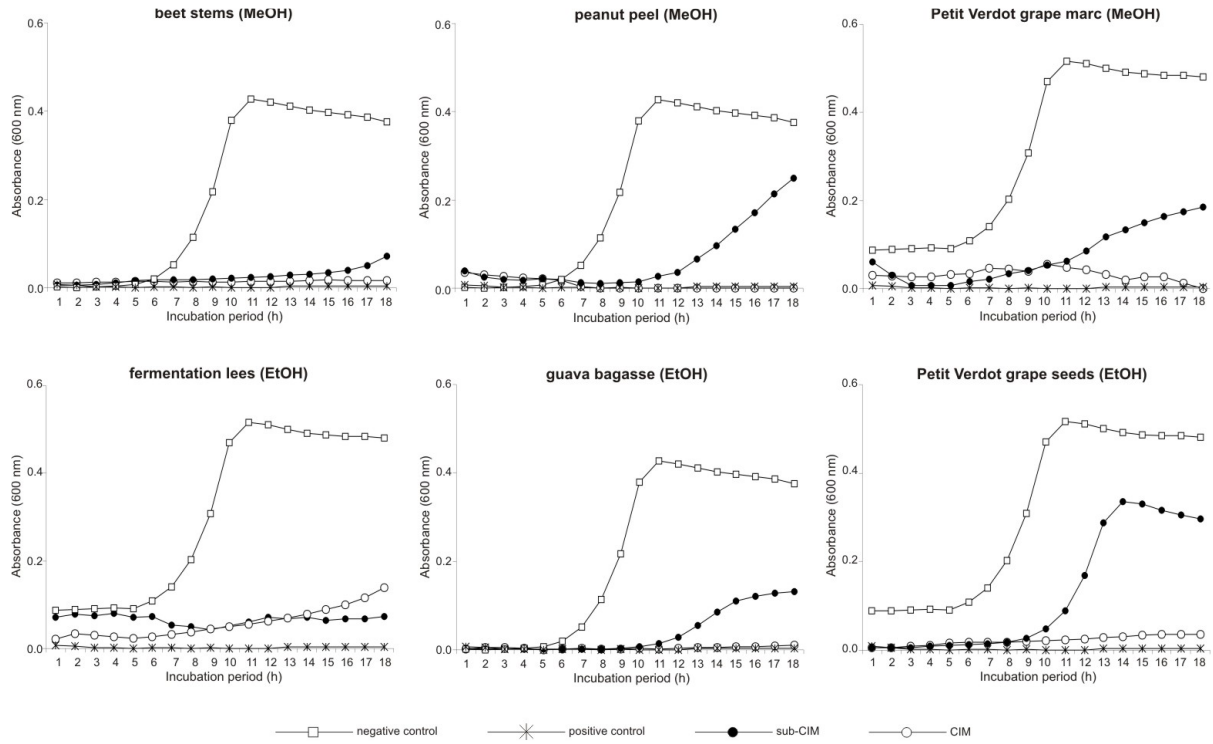


**Figure-2: Growth curves of *L. monocytogenes* in relation to methanol (MeOH) and ethanol (EtOH) extracts of agro-industrial wastes at different concentrations.**