RESEARCH ARTICLE
Bull Biotechnol (2023) 4(2):62-67
https://doi.org/10.51539/biotech.1374327 Journal homepage: www.dergipark.org.tr/biotech

Bulletin of Biotechnology

# Investigation of the antioxidant and antibacterial effects of fermented *Cornus mas* and *Rubus sanctus* fruits

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Received : 11/10/2023
Accepted : 10/12/2023

**Abstract:** In this study antioxidant and antibacterial activities of fermented *Cornus mas* and *Rubus sanctus* berries collected from province of Bartın in the Western Black Sea region of Türkiye were analyzed. Prior to fermentation with *S. cerevisiae,* the fruits were tested for 58 pesticides such as Dicloran and Quintozene and none of the pesticides were detected. The presence of ascorbic acid in the fruits, which is a nutrient needed by the body, was also detected by FTIR. Then the pesticide free berries were crushed, and the samples were fermented separately. Testing after the fermentation process revealed the samples contained ethyl alcohol. Antioxidant activities of fermented samples were analyzed using CUPRAC, DPPH and Folin Ciocalteu methods. The results suggest high antioxidant contents of the fermented samples. Evaluation of antimicrobial activity was done through disk diffusion method using *P.aeruginosa* and *S.aureus* suggesting that these samples do not suppress these bacteria for the studied concentrations. Furthermore, the growth of *C. albicans* was examined immediately, demonstrating that the fermented samples do not show antifungal effects. The reason for these shortcomings could be inadequate concentration levels. The antioxidant content of these fermented fruits is intended to contribute to human health.

**Keywords:** *Cornus mas*; *Rubus sanctus;* Fermentation; Antioxidant; Antibacterial



## 1 Introduction

Throughout life human beings are continuously exposed to factors like radiation, water pollution, environmental pollution, and pesticides etc... As a result of these exposures, free radicals emerge. Free radicals circulate throughout the body to pair their electrons. During this circulation, they cause damage to the body weakening the immune system. Ascorbic acids, thiols, and polyphenols are examples of antioxidants that provide their own electrons to the body's defense against free radical production (Lobo et al. 2010). Fruits and vegetables contain secondary metabolites called phenolic compounds, which have antibacterial properties and inhibit the growth of bacteria, yeast, and viruses (Soong et al. 2019).

Fermented foods provide many health benefits such as antioxidant, antimicrobial, antifungal, anti-inflammatory, anti-diabetic and anti-atherosclerotic activity (Şanlıer et al. 2019).

In this study antioxidant and antibacterial effects of fermented samples of *C. mas* and *R. sanctus* fruit extracts were analyzed. In addition, tests were done to check for pesticides and ascorbic acid content before fermentation and ethyl alcohol formation after fermentation.

## 2 Materials and Method

### 2.1 Obtaining *Cornus mas* and *Rubus sanctus* Fruits

*C. mas* and *R. sanctus* fruits grown in the province of Bartın in the Western Black Sea region of Türkiye were collected ripe in July and August. Fruits were stored in sterilized bags at -20°C.

### 2.2 Determination of Pesticides

Frozen *C. mas* and *R. sanctus* were thawed. At the end of the dissolution period, the fruits were ground using a metal grinder and pre-treatments were applied respectively according to the pesticide analysis protocol. Each pureed fruit sample was diluted and injected into the GC-MS system separately and the analysis results were recorded.

### 2.3 Detection of the Presence of Ascorbic Acid

The pureed fruits were compared with the Ascorbic acid CRS standard with the Agilent Cary 630 FTIR spectrometer. Ascorbic acid is defined in the library of the CRS standard devices. Measurements and comparisons were done after the samples were poured over the device's "crystal" section.

### 2.4 Fermentation

After the frozen fruits were brought to room temperature, they were ground in a grinder and mashed for fermentation. Approximately 20 mg of *C. mas* and *R. sanctus* pureed samples were weighed and taken into four different flasks. *S. cerevisiae,* which was used as the fermenting agent, was added to each sample. 20 ml and 40 ml ultrapure water was added to separate flasks. The samples were sealed, and the fermentation continued for one week. To check for ethyl alcohol presence, the fermented samples were prepared in accordance with the gas chromatography protocol. The prepared samples were injected into the GC-FID device with the ethyl alcohol standard. The results were calculated with the help of the Empower program.

### 2.5 Antifungal Activity Analysis

SDA (Sabouraud Dextrose Agar) medium was prepared and poured into 12 petri dishes (Two concentrations for each fruit extract done in triplicate) and set for 15 minutes to dry. *C. albicans* (ATCC 10231), which is a common fungus, was selected for the analysis. To prepare a suspension from the *C. albicans* strain, it was immersed in the solution in the glass tube with a sterile swab under the biosafety cabinet. Then, the *C. albicans* suspension was dipped into petri dishes. Microorganism suspension was applied to the entire surface of the agar on the petri dishes with a sterile cotton swab. The petri dishes were set aside for 15 minutes to dry. Fermented samples were spread one by one on the dried agars with sterile cotton swabs. Petri dishes were incubated at 20-25 °C and observed for five days (Collins 2004).

### 2.6 Antibacterial Effect Analysis

Fermented samples were studied on *S.aureus* (ATCC 6538) and *P.aeroginosa* (ATCC 9027) bacteria to check for their antibacterial activity (Bayer et al. 1966). Firstly, the bacteria, which were stored as colonies, were taken from the media with a swab with disposable plastic extracts and dissolved in the glass tube in the purchased solution to dissolve the bacteria. The prepared frozen TSA (Tryptic Soy Agar) medium was then dissolved in the ultrasonic bath. The medium, whose dissolution was completed, was poured into 12 petri dishes, and waited for drying. The bacteria-containing solution in the glass tube was applied to the drying media with the help of a sterile cotton swab. The petri dishes were incubated at 20-25˚C for 24 hours. At the end of the incubation period, 8 mm wells were opened in the medium and 50 µl samples were inoculated into the wells with a micropipette. The samples were incubated at 35-37˚C for 24 hours and the results were evaluated.

### 2.7 Antioxidant Activity Analysis

Fermented samples were prepared in accordance with the protocols of CUPRAC, DPPH and Folin Ciocalteu antioxidant capacity analysis methods. The prepared samples were read in the UV-VIS device at wavelengths suitable for the analysis methods and calculated.

#### 2.7.1 CUPRAC Assay

The method developed by Apak et al. was used (Apak et al. 2007). This capacity analysis method named “Copper (II) Reducing Antioxidant Capacity (CUPRAC) involves reduction of the copper-neocuproin complex [Cu(Nc)2]+2 by antioxidant in the presence of ammonium acetate to form the copper-neocuproin complex [Cu(Nc)2]+1. It is a yellow-coloured compound with maximum absorbance at 450 nm. First ammonium acetate buffer is prepared. 19.3 grams of ammonium acetate reagent was transferred to a 250 ml flask and made up to volume with distilled water. Then, 0.01 M copper (II) chloride solution was prepared by weighing 0.43 gr CuCl2.2H2O and made up to 250 ml with distilled water. Lastly, 0.0075 M Neocuproin (2.9-dimethyl 1.10 phenanthroline) solution was prepared by weighing 0.16 grams and making up to 100 ml with ethanol. Gallic acid was used as standard. The samples were prepared in 3 different concentrations by adding 20µl, 40µl and 60µl from the sample solution. The solutions were kept in a dark area for 30 minutes at room conditions. The blank, standards and samples prepared according to the procedure were read by zeroing against the blank at 450 nm and the results were recorded.

#### 2.7.2 Folin Ciocalteu Assay

Fruits and vegetables contain phenolic compounds. They can be oxidized in a basic environment, react with a yellow solution hue, and eventually turn blue. The reason for the formation of this color is that the Folin Ciocalteu reagent is kept in a basic environment. Phenolic substances are measured spectrophotometrically at 700–760 nm with the observation of color change (Lussignoli et al. 1999). Fermented *C. mas* and *R. sanctus* samples with two different concentrations (1 mg/ml and 0.5 mg/ml) were diluted 1/10 with methanol. Samples were vortexed for 30 seconds. Gallic acid was used as standard. 0.5 N Folin reagent was prepared by taking 25 ml of 2 N Folin Reagent from the stock bottle and completing it to 100 ml with distilled water. For 10% Na2CO3 solution, 1 gram of Na2CO3 was weighed to 10 ml, made up to volume with distilled water and mixed. Then, 16 glass tubes were named and put into a tube holder one by one. Blank, standards and samples were prepared with a final volume of 3 ml, respectively.

#### 2.7.3 DPPH Assay

The main feature of the DPPH (2,2-Diphenyl-1-picrylhydrazyl) method is that the transfer of positive charge from the antioxidant substance to the DPPH free radical causes a decrease in absorbance at 517 nm (Sanchez-Moreno et al. 1999). For 0.1 M DPPH radical solution, 39.5 mg of DPPH reagent was weighed and made up to 100 ml with ethanol. The samples were diluted 1/10 with methanol. The blank and samples were prepared by taking 200 µL, 400 µL and 600 µL from the fermented samples, respectively. The prepared solutions were kept in the dark for 30 minutes and read at a wavelength of 517 nm in a spectrophotometer, zeroed against the blank.

## 3 Results

### 3.1 Pre-fermentation Analysis

Pesticide and ascorbic acid analysis were done prior to the fermentation process. 58 pesticides (Table 1) were analysed in the GC-MS device. No pesticide residues were detected in the analysed samples. In the analysis of the presence of ascorbic acid with the FTIR-IR device, the presence of ascorbic acid was determined from the similarity between the wavelengths of 2000-1000 in the chromatograms obtained because of the analysis (Fig 1).

**Table 1** Pesticides analysed using GC-MS

| | | | |
|---|---|---|---|
| Biphenyl | Delta-HCH | Bromophos-methyl | Quintozene |
| THPI | Chlorthalonil | Chozolinate | Tefluthrin |
| Tecnazene | Formothion | Heptachlor exoepoxide | 4,4-DDE |
| Diphenylamine | Ethofumasate 2-Keto | Heptachlor endoepoxide | Dieldrin |
| Ethalfluralin | Parathion-methyl | Captan | Oxyfluorfen |
| Trifluralin | Chloropyrifos-methyl | Folpet | 2,4-DDD |
| Alpha-HCH | Vinclozolin | Procymidone | Chlorfenapyr |
| Hexachlorobenzene | Heptachlor | Chlordane trans | Endrin |
| Dicloran | Fenitrothion | Chlordane cis | Dicofol |
| Beta-HCH | Ethofumasate | Bromophos-ethyl | Chlorthal dimethyl |
| Gamma-HCH | Aldrin | 2,4-DDE | Chlorobenzilate |
| Beta endosulfan | 2,4-DDT | Alpha endosulfan | 4,4-DDT |
| 4,4-DDD | Tetrasul | Chlorfenson | Iprodione |
| Bromopropylate | Tetradifon | Quinoxyfen | Cyhalothrin |
| Methoxychlor | | | |

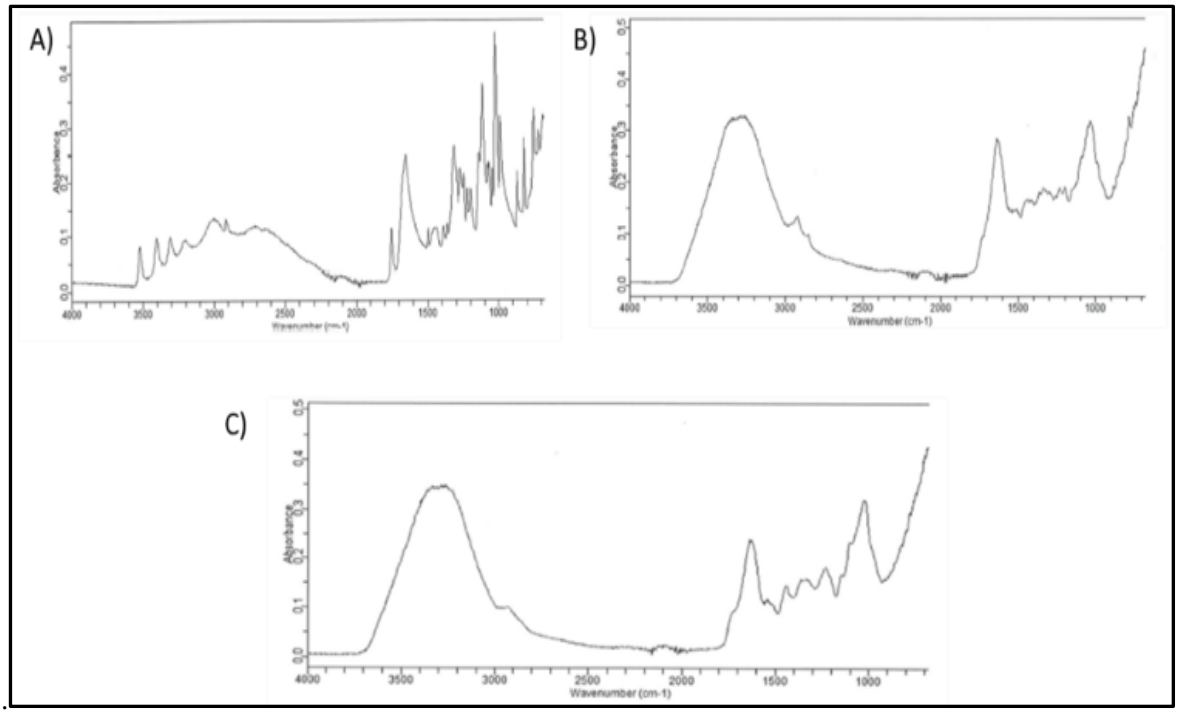


**Fig. 1** A) Chromatogram of standard Ascorbic acid B) Chromatogram of *R. sanctus* C) Chromatogram of *C. mas*

### 3.2 Yeast Ethanol Fermentation

The fermented samples were checked for ethyl alcohol presence. The presence of ethyl alcohol was detected as a result of the analysis. The standard retention time of ethyl alcohol is 4.289. Ethyl alcohol was found in both concentrations of fermented *C. mas* and *R. sanctus* samples (Table 2).

**Table 2** Ethyl Alcohol Determination in the Fermented Fruit Samples

| *Fermented Samples* | *Retention Time* | *Area* |
|---|---|---|
| *Rubus Sanctus- N1* | 4.371 | 14064 |
| *Rubus Sanctus- N2* | 4.329 | 11148 |
| *Cornus Mas*-N1 | 4.395 | 24074 |
| *Cornus Mas*-N2 | 4.364 | 22078 |

### 3.3 Antimicrobial Activity Analysis

In the analysis of the antifungal activity determination of the fermented samples, the growth was observed immediately on the first day, observed up to day 5. Reproduction has increased day by day.

The fermented *C. mas* and *R. sanctus* samples were evaluated for antibacterial activity against strains of *P. aeruginosa* and *S. aureus* microorganisms. Transparent inhibition zones where no growth is seen were identified in the petri dishes at the end of 24 hours (Table 3). The antibacterial activity determination results of the fermented *C. mas* and *R. sanctus* samples are given in image 1.

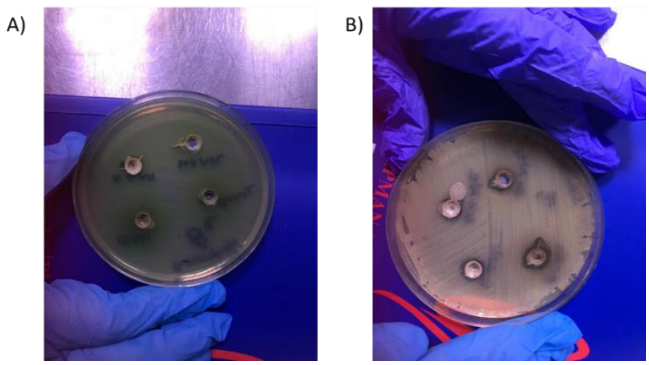


**Image 1** Results of the antibacterial effect of A) *P. aeruginosa* B) *S. aureus*

**Table 3** Inhibition zone diameters of fermented and non-fermented samples against selected bacteria (mm)

| *Samples* | *Unfermented* | *Fermented (average)* |
|---|---|---|
| *Rubus Sanctus*-N1 | - | 10.8 mm*(P. aeruginosa)* |
| *Rubus Sanctus*-N2 | - | 11.1 mm *(S. aureus)* |
| *Cornus Mas*-N1 | - | 11.3 mm *(P. aeruginosa)* |
| *Cornus Mas*-N2 | - | 10.5 mm*(S. aureus)* |

### 3.4 Antioxidant Activity Analysis using DPPH, CUPRAC and Folin Ciocalteu Methods

The antioxidant activity determination analysis results of the samples are given in Fig 2, 3 and 4.

## 4 Discussion

Berry fruits are attracting attention for their beneficial effects on the human gut flora due to their high antioxidant contents. They have preventive advantages on diseases including cancer, cardiovascular, and intestinal (Olas 2018). In our study, the antioxidant, antifungal and antimicrobial effects of the fermented extracts of *C. mas* and *R. sanctus* berry fruits were analysed. The fruits were collected in province of Bartın in the Western Black Sea region of Türkiye. Initially, the fruit samples were examined to determine if there were any pesticide residues present. Since pesticides can have harmful consequences on health, the identification of pesticide residues, especially in foods, has been the focus of many studies (Damalas and Eleftherohorinos 2011). The fruit samples were prepared following protocols and the presence of pesticide residue was checked for 58 parameters in the GC-MS device. There was no evidence of pesticide residue. In addition, the presence of ascorbic acid (Vitamin C), which is an antioxidant and a necessary nutrition for humans (Beyer 1994), was detected in the IR-FTIR analysis before the fermentation of the samples. Previous studies also suggest high ascorbic acid contents for the Rubus genus (Ponder and Hallman 2020). Subsequently, aqueous extracts prepared from *C. mas* and *R. sanctus* fruits were fermented with *S. cerevisiae*. The presence of ethyl alcohol was verified using the GC-FID device. The amount of ethyl alcohol was higher in the fermented samples of *C. mas*. *S. cerevisiae* was able to reproduce in these fruit extracts and could ferment the samples. Song and coworkers have applied *S. cerevisiae* to improve Rubus coreanus Miquel vinegar fermentation process (Song et al. 2019). This application has accelerated the fermentation process.

Antioxidant activity of the fermented samples was analysed using CUPRAC, DPPH and Folin Ciocalteu methods. *R. sanctus* fermented samples at both concentrations showed higher antioxidant capacity values than *C. mas* in the CUPRAC and Folin Ciocalteu analysis results. In the DPPH antioxidant activity determination analysis, the fermented *C. mas* samples with a concentration of 1mg/ml gave higher results than *R. sanctus*, the situation was the opposite in the samples prepared with a concentration of 0.5 mg/ml. The reason for the deviation may be analytical, sample preparation and instrument related. Prior research has also revealed that *Rubus Sanctus* and *Cornus Mas* has greater antioxidant levels (Tiptiri-Kourpeti et al. 2019; Zengin et al. 2020). A very recent research has demonstrated the antioxidant and antimicrobial activity of *C. mas* fruits (Aurori et al. 2023). Additionally, a recent study examined the antioxidant and antibacterial properties of both unfermented *C. mas* fruits and fermented *C. mas* fruits using kombucha (Zagórska-Dziok, 2023). In comparison to the extract, they found that the ferment had greater activity, which supports the validity of employing a fermentation agent may produce valuable products.Though to the best of our knowledge there is no study that involves both fruits.

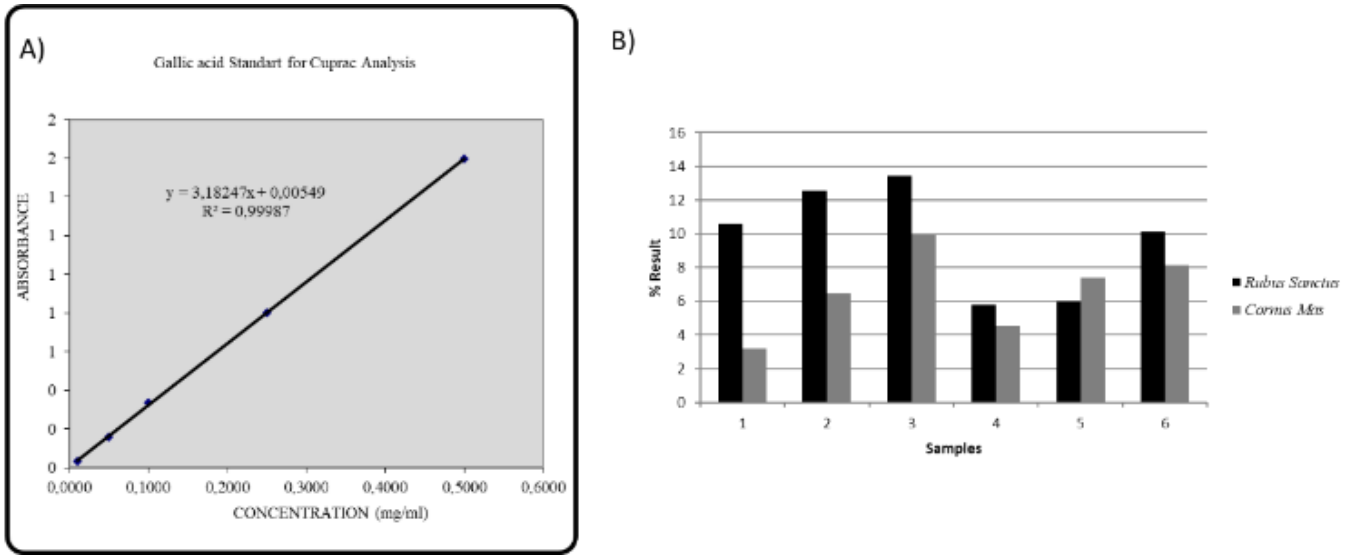


**Fig. 2** A) Linearity study of gallic acid standard B) Graphical comparison of antioxidant capacity results of *C. mas* and *R. sanctus* using CUPRAC method.

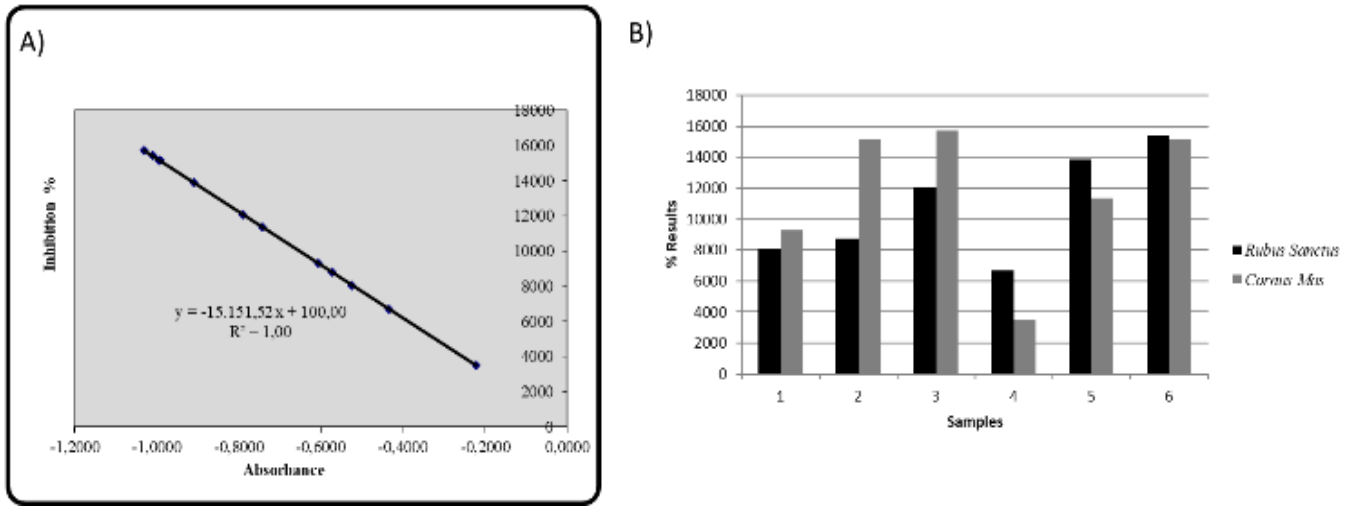


**Fig. 3** A) The inhibition effect of the concentration of antioxidant compounds in the samples on the DPPH solution, B) Graphical comparison of antioxidant capacity results of *C. mas* and *R. sanctus* using DPPH method.

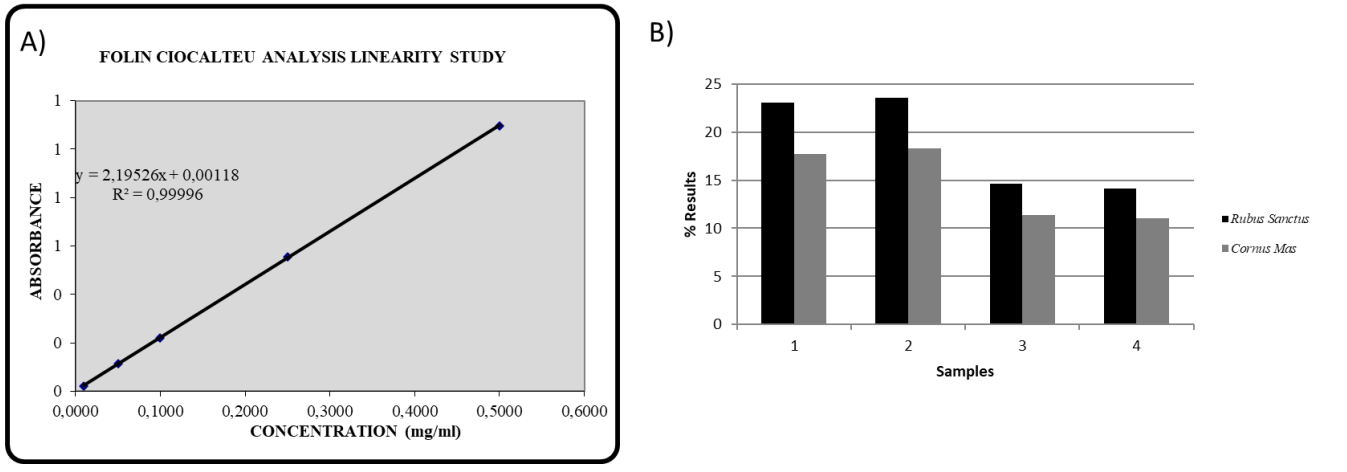


**Fig. 4** A) Linearity study of gallic acid standard, B) Graphical comparison of antioxidant capacity results of *C. mas* and *R. sanctus* using Folin Ciocalteu method

Phenolic compounds found in fruits and vegetables show antibacterial effect by inhibiting the proliferation of microorganisms. *P. aeruginosa* and *S. aureus* were used to check for antibacterial activity, and *C. albicans* was used to check for antifungal activity of the fermented samples. The transparent inhibition zones where no growth is seen is greater in *C. mas* samples. Though Zagórska-Dziok (2023) study has identified high antimicrobial activity with kombucha fermented *C. mas* fruits, in our study the fermented samples do not suppress *P. aeruginosa* and *S. aureu*s greatly. This may be a result of a lower than needed concentration of the fercmented samples. The fermented samples were not effective on *C. albicans* as well, and reproduction begins and continues after 24 hours from the first day.

## 5 Conclusion

Fresh fruit is preserved longer by fermentation, which may allow us to reap their benefits over a longer period. In this study, *R. sanctus* and *C. mas* fruits harvested from the Western Black Sea Region of Türkiye were fermented and their antioxidant and antibacterial activities were analyzed. Fermented *R. sanctus* samples at both concentrations showed higher antioxidant capacity values than *C. mas* in the CUPRAC and Folin Ciocalteu analysis results. Further experiments may be done using different combinations of *R. sanctus* and *C. mas* together or with different fruits, fermenting longer, changing the fermentation steps.

## Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors

## Authors' contributions:

DÜ: data collection, analysis and interpretation of results, and manuscript preparation TS: study conception and design, manuscript preparation, manuscript review

## Conflict of interest disclosure:

The authors have no conflict of interest

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