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ASSESSMENT OF ANTIOXIDANT ACTIVITY OF GEORGIAN CLASSICAL AND KVEVRI-MADE WINE USING THE LUMINOL CHEMILUMINESCENCE METHOD

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კლასიკური და ქვევრში დაყენებული ქართული ღვინის ანტიოქსიდანტური აქტივობის შეფასება ლუმინოლის ქემილუმინესცენციის მეთოდის გამოყენებით

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რეზიუმე

ამ კვლევის ფარგლებში, ჩვენ გავზომეთ ღვინოში არსებული პოლიფენოლებისა და ფლავონოიდების კონცენტრაცია Folin Ciocalteu-სა და $AlCl_3$-ის მეთოდების გამოყენებით და შევაფასეთ ანტიოქსიდანტური აქტივობა ლუმინოლის ქემილუმინესცენციის მეთოდით. ქვევრში დაძველებული ღვინოები ავლენენ პოლიფენოლებისა და ფლავონოიდების მაღალ დონეს. კლასიკურად დაყენებულ წითელ ღვინოში, საფერავში, ამ ნაერთების უფრო მეტი რაოდენობა იყო, ვიდრე ქვევრში დაყენებულ თეთრ ღვინოებში, როგორიცაა ქისი, ხიხვი და რქაწითელი. გამოიკვეთა კორელაცია პოლიფენოლების შემცველობასა და ანტიოქსიდანტურ აქტივობას შორის - მეტი პოლიფენოლის შემცველ ღვინოებს, როგორც წესი, უფრო მაღალი ანტიოქსიდანტური აქტივობა ჰქონდათ. თუმცა, ეს არ ეხებოდა კლასიკურად დაყენებულ ქისს, რომელსაც მეტი პოლიფენოლი ჰქონდა, მაგრამ უფრო დაბალი ანტიოქსიდანტური აქტივობა, ვიდრე რქაწითელს, რაც მიუთითებს ანტიოქსიდანტური ნაერთების ხარისხში არსებულ განსხვავებებზე.

საერთო ჯამში, შეიძლება ითქვას, რომ ქვევრსა და კლასიკურ ქართულ ღვინოში პოლიფენოლებისა და ფლავონოიდების საერთო კონცენტრაცია ნაწილობრივ კორელაციაშია მათ ანტიოქსიდანტურ აქტივობასთან.

1.Introdaction

One of the primary causes of premature aging and many diseases is the action of free radicals. In healthy individuals, natural enzymatic (e.g., SOD, catalase, glutathione peroxidase) and non-enzymatic antioxidant systems counteract oxidative stress. However, under conditions like malnutrition or disease, these defenses may be insufficient, allowing harmful free radicals to accumulate and damage cells [1,2]. Wine, a complex beverage with nutritional and therapeutic properties, owes much of its health benefits to polyphenolic compounds, known for their antioxidant and anti-inflammatory effects. The concentration of these compounds varies by grape variety and production method. Traditional Georgian winemaking in kvevri-large clay vessels-differs significantly from classical methods, potentially resulting in higher polyphenol levels and antioxidant activity due to extended contact with grape pomace and the unique properties of the kvevri [3,4]

This study aims to establish a chemiluminescence-based method to evaluate antioxidant activity and use it to compare polyphenol and flavonoid content, as well as antioxidant capacity, in Georgian wines produced by both traditional and classical methods. It also seeks to determine the correlation between total polyphenol content and antioxidant activity.

2. Materials and methods

2.1. Materials

All chemicals were of analytical purity and were obtained from Sigma–Aldrich.

Determination of the total phenolic content using the Folin–Ciocalteu assay reagents. Gallic acid, aluminum chloride ($ALCL_3$). Quercetin. $NaNO_2$, NaOH. buffer-$Na_2B_4O_710H_2O$, PH=9.18; Potassium ferricyanide (III) 5% solution. 0.05% luminol ($K_8H_7N_3O_2$) solution in dimethylsulfoxide. 3% hydrogen peroxide.

Georgian wines: Rkatsiteli, Saperavi, Kisi, Khikhvi (classical and kvevri wine)

2.2. Methods

2.2.1. Chemiluminescence

Chemiluminescence is the luminescence of bodies, caused by chemical effects or during the course of a chemical reaction. Chemiluminescence is related to exothermic chemical processes. Luminol ($C_8H_7N_3O_2$), a pale-yellow compound, exhibits chemiluminescence when activated by an oxidizing agent. It dissolves well in polar solvents and is widely used to monitor reaction progress and kinetics. In forensics, luminol detects blood through its reaction with iron in hemoglobin. In biology, it's used in western blotting to detect metals and proteins. To produce light, luminol requires an oxidizer like hydrogen peroxide, often catalyzed by substances such as potassium ferricyanide. Under alkaline conditions, luminol forms a dianion, which reacts with oxidized intermediates to form unstable organic peroxides. These rapidly decompose, releasing nitrogen and generating 5-aminophthalic acid with excited electrons. As these electrons relax, light is emitted and recorded via a photomultiplier (diagram #1). The method allows for live observation and studying the kinetics of its progress (diagram #2) [5].

Diagram 1. Schemic diagram of chemiluminometer

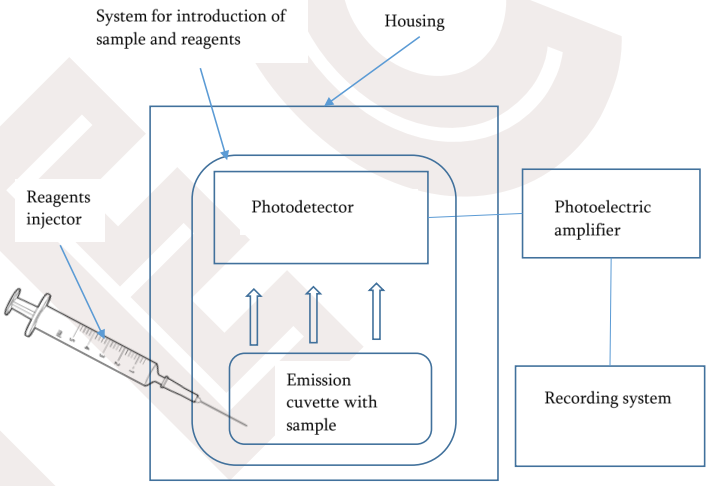
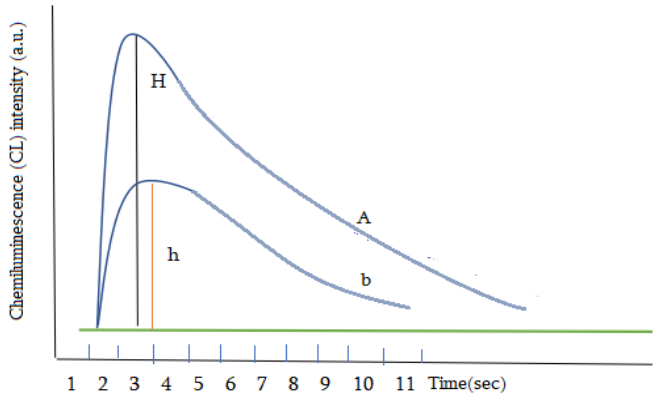


Diagram 2. Conditional graphical representation of the induction curve of luminol chemiluminescence initiated by hydrogen peroxide



The main parameter of the mentioned process on the kinetic curve (A) is the height of the peak H, which is directly proportional to the amount of free radicals, the more radicals are released in the area, therefore, more will come into contact with luminol, oxidize it, and the glow will be correspondingly stronger. In response to this, when an antioxidant is placed in the area, as a result of its action, either the number of radicals directly decreases, or the action of already formed ones is limited, therefore, the brightness decreases and the peak of curve (b) is low - h peak height.

In the currently presented study, during the course of the experiment, we added: 4 ml of buffer- $Na_2B_4O_710H_2O$, PH=9.18, to the incubation area, which is compatible with the chemiluminometer; 5% red blood salt solution - 50µl; 0.05% luminol ($K_8H_7N_3O_2$) solution (we used dimethylsulfoxide as a solvent) - 50µl; We added different samples to the specified mixture, in our case we used: 20-20µl of wines aged in kvevri according to the traditional Georgian method and according to the European classical method: Saperavi, Rkatsiteli, Kisi and Khikhvi. In the control solution, instead of the sample, we added 20µl of alcohol-aqueous solution. Then we transferred the already obtained solution to the luminometer socket and added 200µl of 3% hydrogen peroxide, which was decomposed into various radicals by the action of the red salt of the joint, and their action on the luminol gave us a glow, which was recorded on the self-insect.

First, we calculate the chemiluminescence intensity-peak height of the control solution and wine samples, and express the antioxidant activity by reducing the peak height of the control solution (without antioxidant) by the antioxidant in percent, using the following formula:

$$AA= (1-h/H) \times 100\% \quad (1)$$

H-luminescence intensity-peak height in the area without addition of antioxidant. Height of h-peak after addition of antioxidant. This formula is actively used to express antioxidant activity in EPR data, and we also prefer to use it.

In contrast to the relatively slow chemiluminescence reaction of lucigenin or luminol with solutions under other conditions, in our case the chemiluminescence reaction decays very quickly (diagram #2), while the chemiluminescence reaction of luminol in other studies remains slow and the luminescence intensity becomes maximum after several minutes [6,7].

The main reactive oxygen species produced during degradation of hydrogen peroxide in alkaline solutions are the superoxide radical anion ($O2^{\bullet-}$), the hydroperoxy radical anion ($HO2^{\bullet-}$) and the hydroxyl radical ($OH^{\bullet}$) which react chemiluminogenically with luminol.

2.2.2. Determination of total polyphenols by Folin-Ciocalteu method

The total phenolic content is measured using the Folin–Ciocalteu spectrophotometric method. Phenolic compounds in the sample are oxidized by Folin's reagent, which contains phosphotungstic and phosphomolybdic acids. This reaction produces blue tungsten and molybdenum oxides, and the resulting color intensity is measured at 765 nm. The absorbance, or phenolic coefficient, is proportional to the phenolic content.

Gallic acid is used to create a calibration curve with concentrations of 20, 40, 80, and 120 mg/L. In the experiment, 25 ml flasks were filled with 9 ml distilled water, followed by 1 ml of either sample or gallic acid standard. Wines made from Rkatsiteli, Kisi, Khikhvi, and Saperavi (both kvevri and classical methods) were tested. Next, 1 ml of Folin's reagent was added, followed by a 5-minute wait, then 10 ml of 7% Na_2CO_3. The flasks were topped off with distilled water and incubated in the dark for 90 minutes before measuring absorbance at 765 nm [8].

2.2.3. Determination of total flavonoids by $AlCl_3$ method

This method measures flavonoid content based on the formation of colored complexes with aluminum chloride ($AlCl_3$), which binds to specific hydroxyl and keto groups in flavonoids. The color intensity, read at 510 nm, is proportional to flavonoid concentration. Acid-labile complexes also form with orthohydroxyl groups in flavonoid rings A and B.

A calibration curve is built using quercetin in ethanol at concentrations of 20, 40, 80, 120, and 160 mg/L, with a stock solution of 1 mg/ml. In 25 ml flasks, 10 ml of water and 2.5 ml of either sample or quercetin standard are added; one blank contains only water. Then, 0.75 ml of 5% $NaNO_2$ is added, followed by a 5-minute wait. After that, 0.75 ml of 10% $AlCl_3$ is added, with a 6-minute delay. Finally, 1 M NaOH and 6 ml of water are added, and absorbance is measured at 510 nm [9,10].

2.2.4. Statistical data processing

For the statistical analysis of the data, we used two-way analysis of variance (Two Way Anova). The analysis revealed a statistically significant difference between the antioxidant activity of wines made by different methods (kvevri, classical) ($F=249.743$, $p<0.05$), and between the antioxidant activity of wines made from different varieties of grapes ($F=37.309$, $p<0.05$).

3.Results and discussion

3.1. Concentration of total polyphenols and flavonoids

As expected, kvevri wines showed significantly higher polyphenol concentrations. Among all samples, Saperavi kvevri wine had the highest total polyphenol content at 4070.5 mg/L. Notably, even Saperavi made by the classical method (2605.5 mg/L) had more polyphenols than some white kvevri wines—Khikhvi (1580.5 mg/L), Kisi (2180 mg/L), and Rkatsiteli (2045.5 mg/L). This aligns with known trends, as red wines typically contain more polyphenols due to higher anthocyanin and flavonol levels and differences in production methods.

Table 2. Concentration of total polyphenols in some Georgian wines.

Sample	Concentration of polyphenols (mg/l) (gallic acid equivalent)
Rkatsteli kvevri	2045.25
Rkatsiteli classic	196.75
Kisi kvevri	2180.25
Kisi classic	696.375
Khikhvi kvevri	1580.25
Khikhvi classic	367.75
Saferavi kvevri	4070.5
Saferavi classic	2605.5

A similar ratio can be observed between the content of flavonoids, the Saperavi wine made in Kvevri was characterized by a higher content, which was conditionally assigned 100% content of flavonoids, due to a comparative analysis, and according to this, the percentage distribution of the content of flavonoids of the rest of the wines is given in the form of a table below:

Table 3. Concentration of total flavonoids in some Georgian wines

Sample	Flavonoid content % (quercetin equivalent)
Rkatsteli kvevri	54.3
Rkatsiteli classic	4.9
Kisi kvevri	54.3
Kisi classic	16.3
Khikhvi kvevri	40.7
Khikhvi classic	6.7
Saferavi kvevri	100
Saferavi classic	63

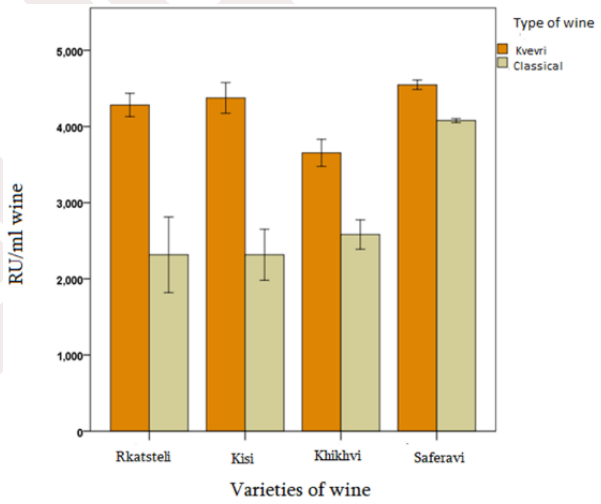
3.2. Antioxidant activity

As we mentioned, we took 20µl of wine samples for analysis and the antioxidant activity was calculated at the mentioned concentrations, however, since it is accepted that the antioxidant activity is expressed in relative units by recalculating per 1-ml sample (in our case, wine), we multiplied the obtained results by the corresponding concentration and presented them in relative units instead of percentage. in the form of a bar chart (see diagram 3).

Using the luminol chemiluminescence method, we calculated the relative antioxidant activity per 1 ml of wine. The highest activity was observed in Saperavi kvevri (4547.5), followed by Kisi (4375) and Rkatsiteli (4282.5), both also made in kvevri. Despite their lower polyphenol and flavonoid concentrations compared to Saperavi, Kisi and Rkatsiteli still demonstrated strong antioxidant activity. This suggests that the qualitative composition of polyphenols—such as the number, position, and stability of hydroxyl groups—plays a key role in antioxidant effectiveness.

Interestingly, Kisi made by the classical method contained nearly four times more polyphenols than Rkatsiteli made the same way, yet their antioxidant activities were nearly identical, further highlighting the importance of polyphenol quality over quantity.

Diagram 3. Relative antioxidant activity represented by bar graph.



According to the two-way analysis of variance (Two-Way Anova), the antioxidant properties of wines placed in kvevri are much higher, regardless of which grape variety they are made from. Antioxidant properties of Saferavi wines are also reliably high, regardless of the technology (Georgian traditional - Kvevari and European - classic) the wine is made. As for the interaction of research factors (type of wine, variety), among them, a statistically significant interaction ($F=18.553$, $p<0.05$) indicates that

although the antioxidant activity of wines made from Rkatsiteli and Kisi grapes does not reliably differ neither in kvevari nor in classic wines, the above-mentioned statistically significant effect ($p<0.05$) of the difference according to varieties is due to the high antioxidant activity of Saperavi in both kvevari and classic wines, The antioxidant activity of wines made with different technologies differs according to varieties: although the antioxidant activity of wines made from Rkatsiteli and Kisi grapes does not reliably differ in either kvevari or classic wines, the reliable difference depending on the varieties is due to the particularly high antioxidant activity of Saperavi in both kvevari and classic wines. Therefore, the antioxidant activity is simultaneously determined by the type of wine, as well as the method of its preparation.

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SUMMARY

Within the framework of this study, we quantified the concentrations of polyphenols and flavonoids present in wine using the Folin Ciocalteu and AlCl 3 methods, and assessed antioxidant activity using the Luminol Chemiluminescence method.

Wines aged in kvevri show high levels of polyphenols and flavonoids. The red wine Saperavi, made classically, had more of these compounds than kvevri-made white wines like Kisi, Khikhvi, and

Rkatsiteli. A correlation between polyphenol content and antioxidant activity was noted-wines with more polyphenols generally had higher antioxidant activity. However, this did not hold for classically made Kisi, which had more polyphenols but lower antioxidant activity than Rkatsiteli, suggesting differences in the quality of antioxidant compounds.

Overall, it can be said that the concentration of total polyphenols and flavonoids in kvevri and classical Georgian wine partially correlates with their antioxidant activity.

Keywords: Free radicals, luminol, antioxidants, chemiluminescence, Georgian wine, total polyphenols and flavonoids.