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Research Article

Use of grape-based stain (Vinatela) on cervical cytology: A Peruvian validation study

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ABSTRACT

Objectives: The Papanicolaou's (Pap's) stain is used for cervical cancer screening. It employs toxic-carcinogenic expensive reagents, which may not be easily accessible to many communities worldwide. The objective of this study was to validate the grape-based alcohol-extracted dye (Vinatela) on normal cervical samples for the Pap test.

Material and Methods: Samples of the two grape species were collected from two vineyards through the Agroindustrial Research Institute of Universidad Privada San Juan Bautista. The dye extraction from the grape species and the dye performance to stain cells were conducted in three phases: (a) direct staining with pre-fermentation wine products, (b) direct fragmentation of grapes and direct staining with shells of the grapes, and (c) alcoholic extraction of the dye. Vinatela obtained from two species (*Vitis vinifera* "*Tempranillo*" and "*Malbec*") and posterior staining of cervical samples. We conducted a double-blind validation on 30 cervical samples.

Results: The basophilic components of the cervical cells were stained. Alcoholic extraction staining protocol had a low yield. The nuclear and cytoplasmic borders, the nuclear details, and the polymorphonuclear nuclei were stained with Vinatela and could be differentiated during nuclear coloration. The initial staining protocol was 10–20 min × (mean ~12 min) staining time. We noted a slightly better staining with *V. v. Tempranillo* as compared to *V. v.* Malbec ($P = 0.045$).

Conclusion: Cervical cells stained with Vinatela stain from two grape species cultivated in the Southern of Peru, showed basophilic nuclear details.

Keywords: Cervical cytology, Grape, Papanicolaou test, Wine industry, Cervical cancer screening

INTRODUCTION

To show the cellular and tissue components, staining techniques are required that allow the differentiation of intrinsic structures, which are not detectable to the human eye. Since cellular components (such as mitochondria and nuclear histones) have different characteristics toward the use of different dyes, due to qualitative or quantitative affinities according to electrical charge, different types of dyes are used (with different auxochromic groups and mordants) for certain cellular structures.[1] If the success of the staining depends on these characteristics, then the

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purpose of the staining is to facilitate the visualization of different structures and to demonstrate their chemical and physical differences between the cellular components.

The Papanicolaou (Pap) stain is a polychromic stain, widely used for the diagnosis of precancerous and cancerous lesions, the estimation of hormonal status, and the status of infections.[2,3] Pap stain is economical, efficient, and fast staining, which has allowed the detection of cervical cancer in high-income countries, dramatically decreasing their mortality rates, and currently constitutes the key piece of secondary prevention of cervical cancer programs worldwide.[4-6] Unfortunately, the current state of many health centers implies few resources to purchase dyes, chemical reagents, glassware, and other supplies in a timely manner.[7] All this add to the toxicity of several of the conventional reagents used during staining. This highlights need to search for faster, simpler, low-cost, and less toxic alternatives for the diagnosis of cervical cancer through exfoliative cytology.[8-10]

From the 1950s to the present day, modifications of the conventional Pap staining have been developed in its different components, stages, and staining time.[8,11,12] Hematoxylin, a nuclear dye, has undergone substantial modifications on its same chemical basis, preventing the use of other plant derivatives for this purpose.[10-13]

The validation of new dyes from other natural sources showing high performance by staining cell nuclei and cytoplasmic components without errors, constitutes a challenge for natural product-research in low-income countries where they require innovation activities for the benefit of Public Health and Global Environmental Health.

We aimed to validate the use of the dye extracted from the grapes (Vinatela) cultivated in the South of Peru, as a staining agent in normal cervical samples.

MATERIAL AND METHODS

Samples of grapes

The present validation study was conducted with two grape species (*Vitis vinifera* "*Tempranillo*" and *V. v.* "*Malbec*"). These samples were collected from two vineyards belonging to the Agroindustrial Research Institute of the San Juan Bautista Private University (UPSJB), located in the South of Peru – Ica Region [Figure 1]. All the samples were collected aseptically by breaking. Grapes with visible damage, rotten or obviously contaminated, were excluded from the study. These were transported to the Research Laboratories of the UPSJB Headquarters in Lima, for the extraction of the dyeing compounds after characterization. This study was approved by the Ethics Committee of the UPSJB (No. 016-2018-VRI-UPSJB.

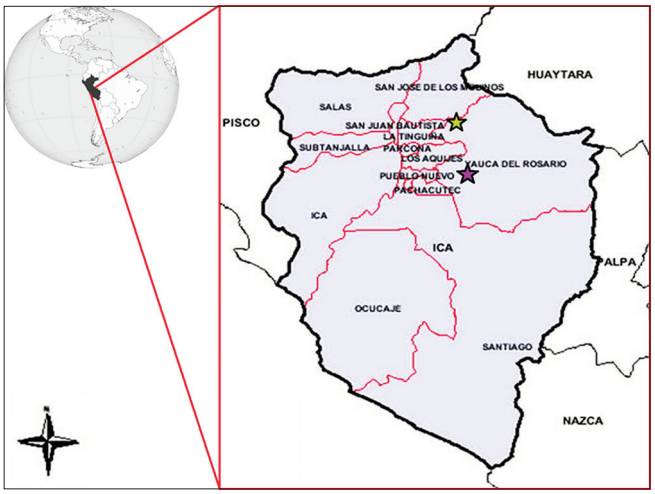

Figure 1: Location of the harvesting areas of the *Vitis vinifera* "*Tempranillo*" and "*Malbec*" grape varieties in Ica, Peru. Fundo 368 (purple star) and Fundo Normita (green star).

Extraction of dye compounds

The extraction of the dyeing compounds from the two grape varieties was carried out in three stages: (1) direct cell coloration tests with pre-fermentation wine products (must) that were obtained from the ARPE winery of the UPSJB, (2) the extraction method by direct fragmentation of grapes, where the shells of the grape species were used directly on the smear,[14] and (3) alcoholic extraction of the dye following the protocol of Hernández (2017).[15] The protocols are detailed in [Figure 2].

For all the extraction products, the physical and chemical characteristics (pH and concentration of wine staining compounds) were evaluated and their nuclear cell affinity capacity (basophilic) was evaluated by microscopy and macroscopy according to previous protocol.[16]

Staining assay

The cell staining tests were performed with the two dye extraction methods and with the pre-fermentation wine products (must) for 10–20 min. The tests were carried out double-blind in 30 normal cervical samples obtained from the Hospital Nacional Docente Madre Niño San Bartolomé following the institution's protocol.[17] Conventional Pap staining was used as a control for nuclear staining and cellular basophilic components.[16] Both colorations were carried out simultaneously. The protocols described in [Figure 2] were applied.

Staining validation protocol

The staining protocols with the wine stain were used to replace the nuclear stain (hematoxylin) during the Pap

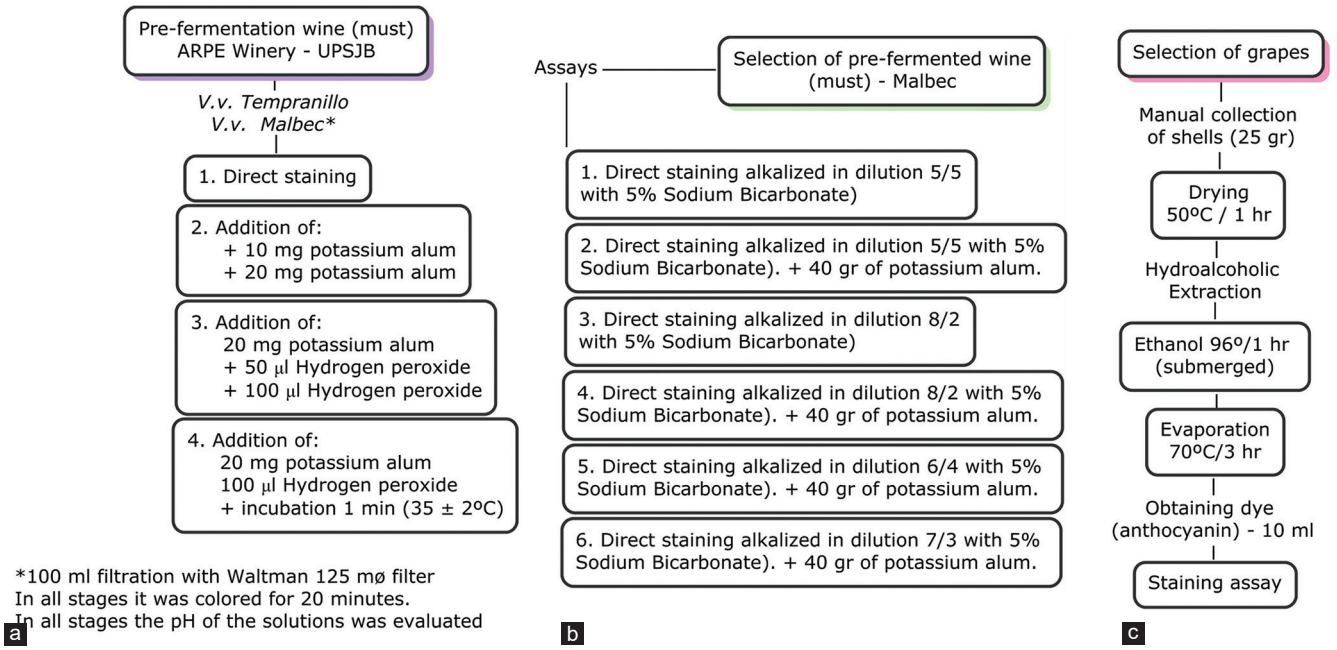

Figure 2: Staining test with Vinatela dye extracted from grape species from Ica, Peru on cervical samples. (a) Protocol 1, direct coloring with pre-fermented wine (must). (b) Protocol 2, coloration test with pH change in pre-fermented wines (must). (c) Hydroalcoholic extraction of "Vinatela" dye.

stain. Cytoplasmic staining processes were performed according to hospital protocol.[16] The quality of staining was evaluated with the staining quality index (SQI) by three cytotechnologists and two hospital pathologists with more than 20 years of experience.[9,10,16,18] Special attention was paid to the basophilic cellular characteristics in the evaluation with the SQI (epithelial cell nuclei, polymorphonuclear cells, and cell border).

Design and statistical analysis scheme

Descriptive and inferential statistics were used to summarize the main findings. For the evaluation of staining quality, the SQI parameters previously described were used.[18] In addition, chi-square was used to establish differences between staining protocols, staining times, and interobserver evaluations. Cohen's Kappa was used to establish the correlation between staining, considering a value of $P < 0.05$ as statistically significant. The data analysis was carried out in the statistical analyzer IBM SPSS v22.0 (Armonk, USA) and EPIDAT-info v2.0 (Galicia, Spain) for MacOS.

RESULTS

The first staining protocol included direct staining and modification of the pre-fermented wine (must), demonstrating a range of effectiveness [Figure 3]. Of the two grape species used in this protocol, the *V. v. Malbec* species showed better performance and stained numerous basophilic structures observed during the macroscopic controls. The use of potassium alum (20 g), hydrogen peroxide (100 µL), and incubation (at 35 ± 2°C) improved the performance in each stage and for both species (*V. v. Malbec* and *V. v. Tempranillo*). After identifying the direct dyeing performance of both grape species, the pH of the solution was changed, showing that the alkalinity of the species improved the affinity of the anthocyanin dyes toward the cellular structures (mainly tests 5 and 7) [Figure 2].

From these tests, we proceeded to extract the peel of both grape varieties, for subsequent analysis and testing of dyeing capacity. In [Table 1], the amounts in grams of grape peel obtained by each species are detailed. On average, 200 g of peel were extracted in 2 kg of grapes. With these shells, the staining capabilities by direct rubbing were compared [Figure 3], showing the greater capacity of the *V. v. Malbec* species. After this test, the dye was extracted, obtaining 4 ± 0.5 mL per grape species (Vinatela solution). With this alcoholic dye, the cervical cytology samples were stained for 20 min, improving the staining capacity [Table 1].

Our results showed differences between direct staining and the Vinatela protocol, the latter being the one that showed

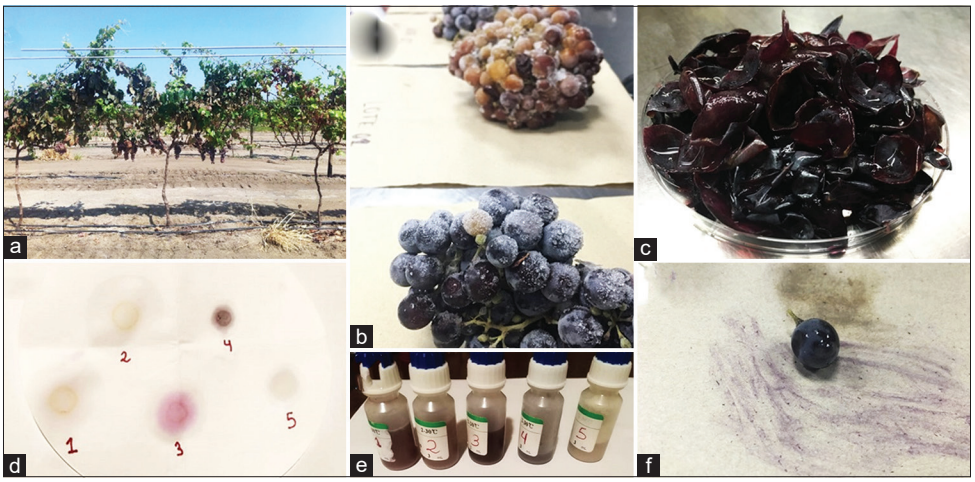

Figure 3: Baseline characteristics of the study. (a) Sample collection sites, UPSJB vineyards, Ica, Peru. (b) Grape species included in the study (*V. v. Tempranillo* -top - and *V. v. Malbec* - below).(c) V. *v. Tempranillo* and *Malbec* grape shells. (d) Evaluation of dyeing capacity on filter paper. (e) Extraction of grape dye under Protocol 3. (f) Comparison of dyeing capacity of grape species by drag. *V. v.*: *Vitis vinifera*.

Table 1: Compounds used in the protocols for staining cervical samples with Vinatela.

S. No.	Solutions
1.	Alcohol 95°+hydrogen peroxide
2.	Hydrogen peroxide
3.	Alcohol 95°
4.	Alkaline solution
5.	Alkaline solution+hydrogen peroxide

the best affinity and dyeing capacity. [Figure 4] shows the control of nuclear staining with each protocol in cervical smears stained with Vinatela. Then, cellular staining characteristics were observed in the nuclear details of both epithelial and polymorphonuclear cells in normal/benign smears [Figure 5]. The mean of the staining procedure was 12 min.

We found better staining characteristics between *V. v. Malbec* versus *V. v. Tempranillo* (P = 0.045). The SQI assessment of staining established an overall SQI of 0.89 (ideal 1). Basophilic component scores showed SQI = 0.90 for chromatic pattern and nuclear staining, SQI = 0.95 for membrane continuity, SQI = 0.80 for background, and SQI = 0.89 for staining overview.

DISCUSSION

This study demonstrated the ability to stain basophilic cellular components with the grape-based stain "Vinatela" on cervical samples from Peruvian women. Of the two evaluated species, our results showed a better performance of *V. v. Malbec*, evidenced in the cellular characteristics evaluated macroscopically and microscopically.

The strengths of this study are supported by the extraction of a stock solution of grape dye (Vinatela) under an alcoholic extraction protocol.[15] As previously described, there is interest in the textile industry in obtaining and using natural colorants extracted from native species of grapes, corn (*Zea mays*), etc.[19-23] The dyes extracted from these species represent a great economic contribution to the industry of different countries, producing an annual investment of 500 million dollars in Latin America.[14] In this sense, our findings contribute to the research in the extraction of natural dyes and exceed this previous purpose using it as a cell stain of basophilic structures. Thus, it could be used in routine clinical and pathological laboratory stains (Pap stain, basophilic stain in the carbolfuchsin technique for the determination of sex chromatin, wright for blood smears, Gram for bacteria staining, etc.). This topic requires future application research.

The limitations of this study are mainly due to the nature of its design. This is the first study with an exploratory approach that has focused its methodological development on first achieving the extraction of the grape stain, and second, demonstrating its affinity toward the basophilic components of the cervical cellular structures being able to estimate their staining quality in the macro and microscopic evaluation.

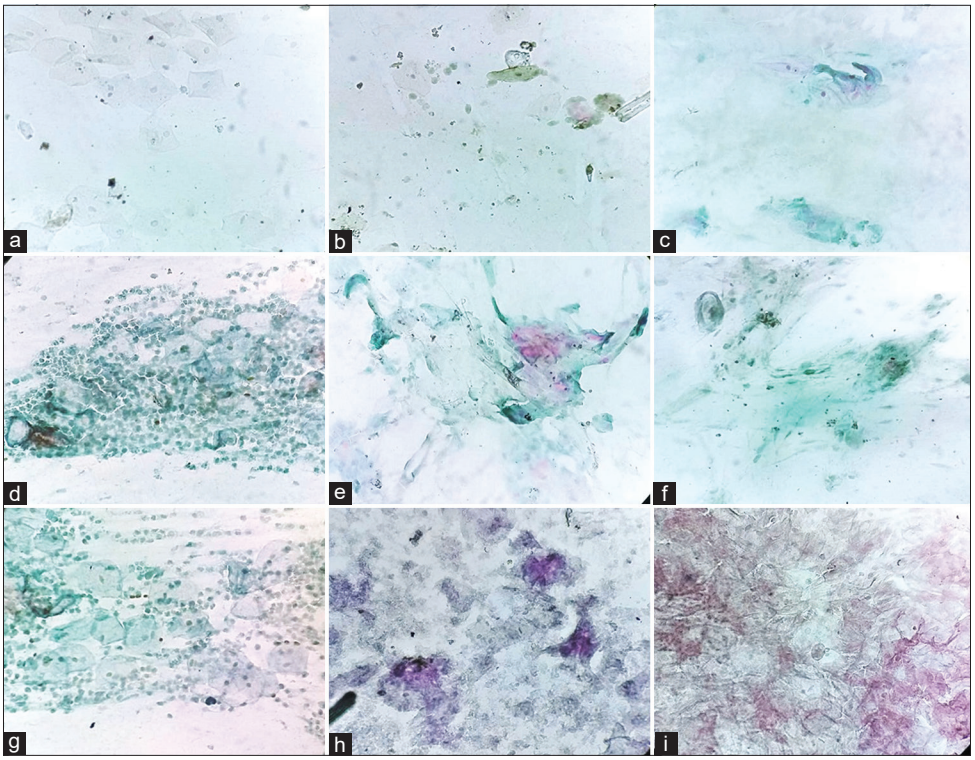

Figure 4: Photomicrographs of cervical smears stained under the three staining protocols. With protocol 1 (a-d), no staining of the cellular components was obtained with *V. v. Tempranillo* (a and b), with *V. v. Malbec* slightly higher staining results were obtained (c and d). With protocol 2, improvements in staining capacity were obtained mainly in the 5:5 dilution (e and f) with *V. v. Malbec*. Protocol 3 (Vinatela) showed better cervical cell staining capacity (g-i), the staining capacity was slightly higher with *V. v. Malbec* (h and i). *V. v.*: *Vitis vinifera.*

In this sense, the Vinatela stain obtained from *V. v. Malbec* demonstrated an optimal global SQI (0.89), being comparable with the prolonged Pap modification (SQI = 0.94), with the ultrafast Pap staining (quality index = very good), with the Cytocolor (Szczepanik modification) (SQI = 0.87), with the short Pap's eosin-thiazine stain, and with the ecological stain of Pap (Eco-Pap) (SQI = 0.94).[9,10,16,18,24,25]

On the other hand, the development of natural colorants that can be used in pathology laboratories is part of the Sustainable Development Goals (SDG) of the United Nations for 2030.[26] Efforts for laboratories to reduce their high proportion of daily pollutants are one of the commitments that laboratories and environmental health control organizations must prioritize since tons of toxic pollutants with high carcinogenic potential are eliminated daily causing a carbon footprint[27] and play an irreplaceable role as submarine emissaries (destroying the marine life that irreparably worsens the depressed state of the current climate).

As we proposed with the creation of the Eco-Pap,[10] the development of laboratories specialized in natural colorants is required, which through technology transfer could reduce the impact of environmental pollution and boost the bioeconomy of the countries of the region. This progress in the study of natural dyes cannot only address the SDGs (meeting 6 of the 17 objectives), but through its development, it contributes to improving Global Environmental Health and being able to be incorporated into the routine flow work of laboratory (mainly in the Pap, which is used as the main mass secondary prevention tool) could improve cervical cancer screening (which has increased in the past 6 years)[28] contributing to Public Health.

Vinatela initiates a new scope of action for components derived from grapes. The textile and wine industry already generates an income, being able to benefit from the possible use of grape-based stains within pathology laboratories. In this study, native grapes were used and grown in the UPSJB vineyards, highlighting the grape species from southern Peru.

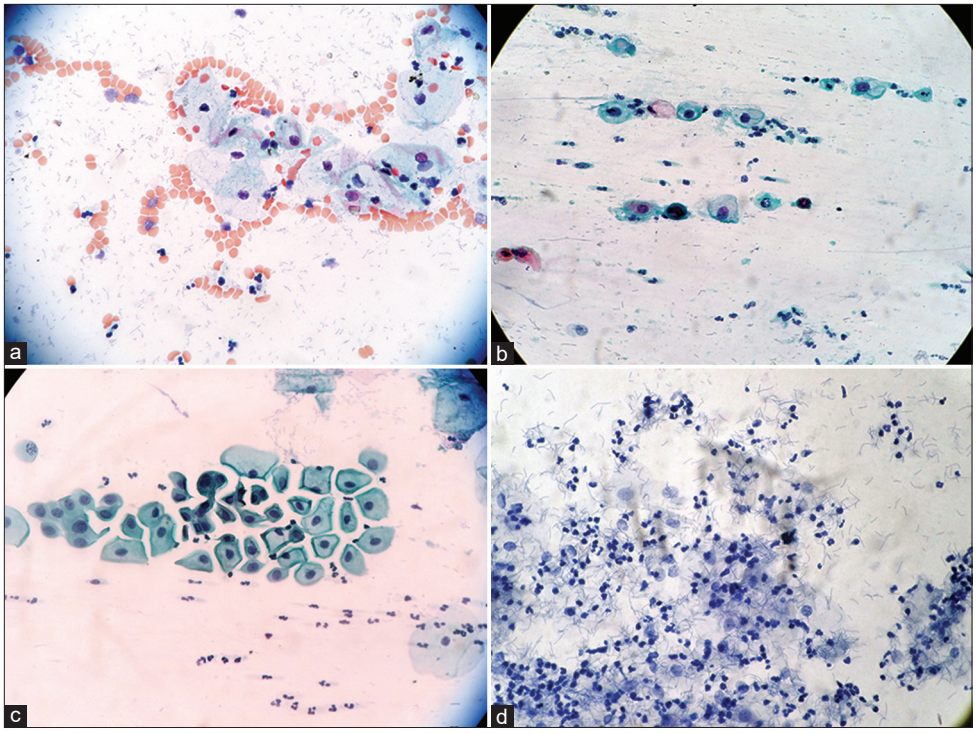

Figure 5: Photomicrographs of cervical smears stained with Vinatela-based-*V. v. Malbec.* (a) Negative for intraepithelial lesion or malignancy (NILM), intermediate squamous cells with surrounding peripheral erythrocytes. (b) NILM, navicular cells. (c) NILM, metaplastic squamous cells. (d) NILM, shift in flora suggestive of bacterial vaginosis. *V. v.*: *Vitis vinifera.*

SUMMARY

We validated the Vinatela nuclear stain, from grapes grown in Peru through the alcoholic-extraction protocol, in normal cervical samples as Pap test with optimal staining quality.

COMPETING INTERESTS STATEMENT BY ALL AUTHORS

The authors declare that they have no competing interests.

AUTHORSHIP STATEMENT BY ALL AUTHORS

All authors of this article declare that we qualify for authorship as defined by ICMJE.

Each author has participated sufficiently in the work and takes public responsibility for appropriate portions of the content of this article.

Jeel Moya-Salazar planned the study design and carried the out the sampling, staining, image analysis, and alignment and drafted the manuscript. Víctor Rojas-Zumaran planned the study design and carried the out the sampling, staining, image analysis, and alignment and drafted the manuscript. Carlos Vegas planned the study design and carried the out the sampling, and drafting and alignment of the study. Amalia Salafia and Hans Contreras-Pulache play a role in the drafting and alignment of the study. Hans Contreras-Pulache has contribution to statistical analysis.

All authors read and approved the final manuscript.

Each author acknowledges that this final version was read and approved.

ETHICS STATEMENT BY ALL AUTHORS

This study was conducted with approval from the Institutional Ethical Committee. Authors take responsibility to maintain relevant documentation in this respect.

LIST OF ABBREVIATIONS (In alphabetic order)

SQI: Staining Quality Index
UPSJB: San Juan Bautista Private University
V. v. "*Tempranillo*": *Vitis vinifera* "*Tempranillo*"
V. v. "*Malbec*": *Vitis vinifera* "*Malbec*"
Vinatela: Grape-based stain.

EDITORIAL/PEER-REVIEW STATEMENT

To ensure the integrity and highest quality of CytoJournal publications, the review process of this manuscript was

conducted under a **double-blind model** (authors are blinded for reviewers and vice versa) through the automatic online system.

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