**Red and white wine lees show inhibitory effects on the liver carcinogenesis.**

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**Abbreviations:**

HCC: Hepatocellular carcinoma

GAE: Gallic Acid

HPLC: High-performance liquid chromatography

DEN: Diethylnitrosamine

HE: Hematoxylin & Eosin

**Keywords:** Wine lees, DNA methylation, diethylnitrosamine, hepatocarcinogenesis.

**Abstract**

**Scope:** Wine has shown anticarcinogenic benefits in hepatocarcinoma and polyphenols seem to be responsible for these effects. Wine lees are the sediments produced during fermentation and they endow wine with organoleptic and physicochemical properties. However, the anticarcinogenic role of these compounds is still unknown. Thus, the purpose of this work was to determine the phytochemical profiles of wine lees and then, to analyze their anticarcinogenic effect and DNA methylation on a model of hepatocarcinogenesis.

**Methods and results**: The phytochemical composition of lees was determined by Folin-Ciocalteu method and by High–Performance Liquid Chromatography. An *in vivo* study using a diethyl nitrosamine hepatocarcinogenesis-induced model was performed to investigate the hepatoprotective properties of different doses of wine lees. For the DNA methylation analysis a bisulfite-based method was used. Both types of lees mostly contained pyrogallol, gallic and syringic acid with a high content of catechins in red lees. The carcinogen hypermethylated the Alu-M2 repetitive sequence and white lees decreased the hypermethylation at all tested concentrations. Low concentration of red and white lees and high concentration of white lees, significantly improved the hepatocellular architecture and decreased the mitotic index in the murine model.

**Conclusion:** These findings suggest that wine lees are promising agents for chemoprevention of hepatocarcinoma.

## 1. Introduction

Hepatocellular carcinoma (HCC) is among the most common neoplasias representing the fifth most common malignancy worldwide, and the second most common cause of cancer-related deaths in the world.[1,2,3] Cirrhosis is the strongest and the most common risk factor for HCC, usually owing to hepatitis B/C virus infection but some other etiologies such as obesity, fungal metabolites (aflatoxin B1) and chemical carcinogens are also emerging in the last years. [1,4] The incidence of this neoplasia is increasing, and the increase is expected to continue in the future as a result of the rise of chronic hepatitis C. [5]

Currently, surgical resection and liver transplantation are the main curative therapies for early-stage HCC tumors. However, at more developed stages of the disease, chemoembolization and sorafenib, a multikinase inhibitor, are the only strategies which have shown survival benefits for the patients. [3] Recent advances in molecular classification of HCC for therapy stratification are under investigation to define novel and more encouraging therapeutic approaches; nevertheless, these promising approaches are very limited at present. In the meantime, there is an urgent need for alternative therapeutic strategies with improved potency on HCC.

Recently, chemoprevention with the use of natural or synthetic chemical agents has demonstrated substantial protective properties for this malignancy and they have proved to be less toxic than conventional therapies. [2,6,7] Thus, several promising chemopreventive agents such as statins or metformin and dietary agents (hesperidin, coffee, vitamin E and fish oil) have shown their efficiency for this malignancy. [3,8,5,9,10] Several epidemiological and preclinical studies have also shown that phenolic extracts from wine possess potent antioxidant, anti-inflammatory and antineoplastic properties in liver cells and thus, these wine components could constitute an attractive chemotherapeutic option for these patients. [5,9,11,12,13,14] The anticarcinogenic potential of phenolic extracts from wine is based on their antiproliferative activity and ability to induce apoptosis in several cancers. Wine polyphenols scavenge free radicals, thus reducing and repairing oxidative damage of DNA, proteins, and lipids.[15,16] While most studies have investigated the anticancer effects of wine polyphenols, some other wine compounds have not been extensively studied for their anticancer or cancer preventive activity. Much effort has been devoted to characterize and understand the complex composition of wine with the aim of finding new chemopreventive extracts. Thus, it seems essential to investigate the anticancer effects of other wine components looking for new sources of bioactive compounds.

Wine lees is the name given to the sediments resulting from the precipitate formed during the wine fermentation and mainly consists of the dead yeast cells, grape skin, seed fragments and various grape solids. Lees influences the structural integration of the wine in terms of body, flavor, oxidative buffering and wine stability. Therefore, although it is well known that wine lees have potential organoleptic benefits, their anti-tumor activity have not been previously explored.

Here, we provide an *in vivo* evaluation of wine lees activity on HCC.. Our study provides preclinical evidence of the anticarcinogenic effect of wine lees on HCC.

## 2. Experimental Section

### *2.1 Chemicals and Preparation and Characterization of Extracts from White and Red Wine Lees*

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). All the chemical determinations and characterizations were carried out in the laboratory of metabolomics/proteomics and exploitation of Agrifood residues of the University of Córdoba (FQM-227).Wine lees were obtained by raking after alcoholic fermentation from Syrah and Pedro Ximénez grapes, respectively (Cooperativa Agrícola "La Unión", Montilla, Córdoba, Spain). The lees were centrifuged at 2100×g and the liquid phase was discarded. The solid phase was used to obtain the extracts. The extraction of the target compounds from lees was performedwith 100 mL of 60% (v/v) aqueous ethanol at pH=4, and using microwave irradiation at 140 W for 10 min. The extracts were dried in a rotary evaporator to a quarter pf their initial volume to remove ethanol, then centrifuged for 10 min at 855.27×g to separate the solid residue. Finally, each concentrated extract was filtered using a 0.45 µm filter before injection into the chromatograph (Varian, Palo Alto, California, USA). Data processing was carried out using a Star Chromatography Workstation version 5.52 software.

### *2.2 Determination of Total Phenols by the Folin–Ciocalteu (F–C Method.*

The amount of total phenolic compounds was measured by the F–C method [17] using gallic acid (GAE) as calibration standard. The calibration curve was carried out with solutions of 100, 200, 300, 400, 500 and 600 mg/L of this compound ($y = 0.0009x + 0.0081$, $R^2 = 0.9978$). A 0.5-mL aliquot of extract, 10-mL distilled water, 1-mL F–C reagent and 3-mL $Na_2CO_3$ (20%, w/v) were mixed, made to 25 mL with distilled water and heated at 50 ºC for 5 min. After heating, the samples were kept at room temperature for 30 min and, finally, the absorbance was measured at 765 nm against a blank solution containing distilled water instead of extract. The concentration of phenolic compounds thus obtained was multiplied by the dilution factor of the extract volume and divided by the amount of lees used. The results were expressed as equivalent to milligrams of GAE per g of lees extract (mg GAE/g lees).

### *2.3 Determination of Total Anthocyanins by the Page Method.*

The amount of total anthocyanins was measured by the Page method [18] using peonidin-3-glucoside as calibration standard. The calibration curve was carried out with solutions of 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg/L of this compound ($y = 0{,}0306531x + 0{,}00139968$, $R^2 = 0.9986$). A 0.5-mL aliquot of extract and 10-mL distilled water were mixed, made to 25 mL with distilled water and, the absorbance was measured at 535 nm against a blank solution containing distilled water instead of extract. The concentration of anthocyanins thus obtained was multiplied by the dilution factor of the extract volume and divided by the amount of lees used. The results were expressed as equivalent to milligrams of peonidin-3-glucoside per g of lees extract (mg peonidin-3-glucoside/g lees).

### *2.4 High-Performance Liquid Chromatography (HPLC) Separation and Diode Array Detection (DAD) for Analysis of Red and White Wine Lees.*

The lees components were separated by HPLC and determined by a diode array detector (DAD). Separation of the analytes in the extracts was performed on an Inertsil ODS-2 column (250 mm × 4.6 mm i.d., 5 µm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain), using an injection volume of

20 µL and a flow rate of 1 mL/min. The mobile phases consisted of 0.2% (v/v) phosphoric acid aqueous solution (phase A) and methanol (phase B). The gradient was as follows: from 96% to 82% A in 20 min, held for 20 min, from 82% to 74% A in 24 min and from 74% to 50% B in 9 min. The analytes were identified by comparing both their retention times and ultraviolet spectra with those of the corresponding standards. The absorption wavelengths were set at 280 nm for monitoring hydroxybenzoic acids, and at 320 nm for catechin. The analyses were performed in triplicate.

*2.5 In vivo Study. Animals and Diet.*

Forty-four pathogen-free male Sprague-Dawley rats (357 gr±18 gr), seven weeks old, were supplied from Harlan Interfaunan (Iberica) S.L and acclimatized for a week with controlled temperature (23º±2ºC) and humidity (55%±5%). The animals were fed with a standard diet (D03- SAFE, Augy, France) and provided with drinking water *ad libitum*. Animal care and experimental procedures were approved by the University of Cordoba Bioethics Committee, and followed the regulations of the European Union normative for care and use of laboratory animals.

*2.6 Experimental Design*

To evaluate the *in vivo* hepatoprotective effects of wine lees, the effect of wine lees on a Diethylnitrosamine (DEN) induced rat model of hepatocarcinogenesis was first tested. After acclimatization, the rats were weighed and randomized into eight groups for each experiment, one experiment with red wine lees, and another experiment with white wine lees, as follows: 1) Negative control group (rats with standard diet and water *ad libitum*). 2) Positive control group (rats with standard diet and DEN diluted at 0.01% in drinking water). 3) Group 1 (rats with standard diet supplemented with 4000 ppm of lees (red or white lees). 4) Group 2 (rats with standard diet supplemented with 2000 ppm of lees (red or white lees). 5) Group 3 (rats with standard diet supplemented with 1000 ppm of lees (red or white lees). 6) Group 1 + DEN (rats with standard diet supplemented with 4000 ppm of lees (red or white) and 0.01% DEN in drinking water). 7) Group 2 + DEN (rats with standard diet supplemented with 2000 ppm of lees (red or white) and 0.01% DEN in drinking water). 8) Group 3 + DEN (rats with standard diet supplemented with 1000 ppm of lees (red or white) and 0.01% DEN in drinking water). Feed and water consumption for each animal was monitored daily. No differences in intake were observed between control and experimental groups. A schema of the study is provided in the Figure 1.

*2.7 Dosage information/Dosage regimen*

The negative control group were fed with a standard diet and provided with drinking water *ad libitum* during all the experiment. For the administration of wine lees, the corresponding dose of wine lees (4000, 2000 or 1000 ppm) was added daily with a sweetened jelly to ensure palatability and the whole intake of the lees. This alternative dosing method has proven to be adequate and effective in previous studies with rats. [19] Regarding administration of DEN, it was diluted at 0.01% in drinking water since it has proven to be the most common and least stressful dosing method for the DEN [9,10,11]

*2.8 Hepatic Histopathological Evaluation*

At the end of the study (12 weeks), the rats were weighed and sacrificed by $CO_2$ inhalation and subsequent decapitation. Immediately after sacrifice the livers were perfused through the portal vein with

saline solution and subsequently removed, weighed and minutely examined grossly for the presence of visible hepatocyte nodules of varied sized and noted the percentage of parenchyma affectation. Measurements of size and number of nodules were done in two perpendicular planes to obtain an average diameter of each nodule and categorized into three groups (≥3, <3->1 and ≤ 1 mm) according to Bishayee and Dhir (2009). [209] For microscopy studies, representative sections from right, left and caudate lobes of each liver were taken, as well as of the largest lesions found, and fixed in buffered formalin (10%), embedded in paraffin wax and stored at 4ºC. The sections were stained with hematoxylin and eosin (HE). Hepatic lesions were classified by light microscopy by two different exposure-blinded pathologists according to the guidelines proposed by Thoolen et al. (2010). [21] .Briefly, each sample was evaluated for the presence of foci of cellular alteration and other liver injuries such as necrosis, cholestasis, inflammatory infiltrate, bile duct hyperplasia, oval cell hyperplasia and regenerative hyperplasia. Additionally, mitotic figures were counted in 10 non-overlapping high power fields per sample. Also, five µm thick sections of liver samples were prepared for Masson´s trichrome (Sigma, USA) staining as a marker for detecting the degree of liver fibrosis. Examination of the slides was performed a light microscope according to the criteria established by Batts et al. (1995) to determine the liver injury for each group. [22]

*2.9 DNA Extraction and Methylation Analysis of ALU-M2 Repetitive Sequence.*

Portions of the liver samples were frozen in liquid nitrogen and stored at -80ºC until DNA extraction. Genomic DNAs were extracted from the liver samples using a commercial kit (MBL 243, Dominion mbl, Córdoba, Spain) following the manufacturer instructions. Genomic DNA (1µg) was denatured with NaOH and modified with sodium bisulfite using a CpGENOME$^{TM}$ DNA modification kit (Chemicon International, Temecula, CA), following the manufacturer´s recommendations. DNA treated with bisulfite converts unmetylated cytosine residues to uracil, but leaves 5-methylcytosine residues unchanged. Therefore, bisulfite DNA treatment retains only methylated cytosines. Then methylation-specific PCR was proceeded. Briefly, the repetitive sequence of Alu-M2 (Forward: GCGCGGTGGTTTACGTTT and Reverse: AACCGAACTAATCTCGAACTCCTAAC) was used as a surrogate marker to estimate global DNA methylation and Alu-C4 was used as housekeeping gene for control of the reaction (Forward: GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA and Reverse: ATTAACTAAACTAATCTTAAACTCCTAACCTCA).

The qrt-MSP reaction was performed on a Light-Cycler$^{TM}$ instrument system (Roche, Mannheim, Germany) using 1µL of bisulfite-modified DNA in a final reaction mix volume of 10 mL with 0.4 mmol/L of each primer, and 1 mL of 10_LightCycler FastStar DNA Master SYBR Green I (Roche Molecular Biochemicals). The final concentration of $MgCl_2$ for each reaction mixture was 3.5 mmol/L. Denaturation proceeded at 95°C for 10 min (1 cycle) and followed by 45 cycles at 95°C for 10 s 65°C for 10 s, and 72°C for 10 s. Briefly, melting program of 40ºC for 60 s (1 cycle) and a final cooling program of 4ºC for 60 s (1 cycle). The temperature ramp rate was 20ºC/s, except in the melting program, which was 0.2ºC/s between 40 and 95ºC. The calculations were done by relative quantification and automatically obtained by the software LightCycler (RealQuant, version 1.0 Roche). Each sample was analyzed by triplicate.

*2.10 Statistical Analysis*

For statistical data evaluation, the SPSS 15.0 statistics software (SPSS Inc. Headquarters, Chicago, IL, USA) was used. Differences between the means of rat groups were assessed by an ANOVA and the Duncan post-hoc test was applied. Differences between the means of repetitive sequences methylation pattern were assessed by an ANOVA and the Tuckey post-hoc test was applied. Differences were considered statistically significant when $p \leq 0.05$.

## 3. Results

*3.1 Characterization of Red and White Wine Lees*

The phenol and anthocyanin profiles from red and white wine lees are listed in Table 1. Catechin was the principal component in red wine lees whereas in white wine lees no catechin was found. In both wine lees, pyrogallol, gallic, and syringic acids were present in notable quantities but in higher proportions in white lees . In general, red wine lees have a total phenolic content 3.5 times higher, and an anthocyanin total content 80 times higher than white wine lees. The phenolic profile differs essentially as catechin represents 90% of the phenolic content of red wine lees, while it is absent in white wine lees.

*3.2 Effect of Lees on the Body and Relative Liver Weights*

No differences were found in the average body weights and liver weight of any of the single treatments with lees and the negative control group, suggesting that red and white wine lees did not interfere with the animal´s growth (Table 2). On the other hand, DEN treatment drastically decreased the body and liver weights . Co-treatment with DEN and lees increase the final body weight of the rats with the lowest concentration for red wine lees (Group 3 + DEN) and highest concentration for white wine less (Group 1 + DEN). No concluding results could be obtained from Group 1 (4000 ppm of lees) + DEN in red wine lees owing to the sudden death of two of the rats.

*3.3 Effect of Red and White Lees on Liver _Nodules Growth*

There was no macroscopic liver nodules growth in the liver of the negative control group or in red and white wine lees control groups (Table 2). The hepatic parenchyma was apparently normal and with no alteration of the normal color (Figures 2A and 3A). However, macroscopic liver nodules were significantly found in DEN-exposed group (Figures 2B and 3B). Most of the liver nodules varied from white to gray-white color and the size was between 1.0 mm to 3.0 mm (>95% of the nodules) but with some them of 8.0 mm. Comparing with the DEN group, rats that received DEN + 2000 or 1000 ppm of red wine lees significantly underwent a decreased liver nodule growth (27% and 38% of nodule growth reduction respectively) (Figures 2G and 2H). Concerning white wine lees, rats with a supplement of 1000 and 4000 ppm of white wine lees displayed a potent decrease of liver nodule growth (70% and 90% of nodule growth reduction respectively) (Figures 3H and 3F).

*3.4 Effect of Red and White Lees on Hepatic Histology*

In both experiments, negative control group and red and white wine lees control groups (Groups 1, 2 and 3), showed the typical lobular architecture of polyhedral hepatocytes with granular cytoplasm and small uniform nuclei arranged in cords and with a mitotic index of 0-1 in 10 high-power fields (Figures 2A and

3A). By contrast, livers from animals exposed to DEN presented complete loss of normal architecture, with irregular shape of hepatocytes and enlarged and hyperchromatic nuclei and with a mitotic index of 8-9 in 10 high-power fields (Figures 2B and 3B). Several high grades of hepatic lesions were observed in the DEN-treated group: foci of cellular alterations, bile duct hyperplasia, oval cell hyperplasia, regenerative hyperplasia, cholectasis, hepatic necrosis, focal fatty change and inflammation. The main found lesion was foci of cellular alteration composed of usually enlarged, polygonal hepatocytes with acidophilic staining cytoplasm from the surrounding normal parenchyma (Figures 2B and 3B). In addition, one rat from the DEN group developed cholangiofibrosis consisting of dilated to cystic bile ducts and surrounded by inflammatory cell infiltrates and abundant connective tissue Supplementation of 1000 ppm of red wine lees resulted in significant improvement of liver histology as compared to DEN group (Figure 2H).Supplementation

Regarding the experiment with white wine lees, rats fed with 4000 or 1000 ppm of white wine lees and DEN showed significant improvement of hepatocellular architecture with more regular and less altered hepatocytes, and lower mitotic index when compared to the DEN group. In short, hepatic lesions were less frequent and severe. This improvement of hepatocellular architecture was most evident in rats that received white wine lees at 1000 ppm (Figure 3H).

*3.5 Determination of Hepatic Fibrosis*

The degree of fibrosis determined by Masson´s trichrome staining of the liver sections from all groups is shown in Figure 4. Liver sections from control groups appeared normal without signs of fibrosis. Liver sections from DEN group revealed increased deposition of collagen fibers around lobules and portal spaces indicating severe fibrosis. Livers from rats treated with high and low doses of white lees showed moderated deposition of collagen fibers while those from rats treated with any concentration of red lees showed no differences in fibrosis.

*3.6 DNA Global Methylation Effects in the Liver by Red and White Wine Lees.*

Figure 5 shows the methylation status of ALU-M2 sequence in rat liver at different concentrations (1000, 2000 or 4000 ppm) of white and red wine lees when compared to the negative control group. The rats fed with white wine lees had no significant differences in genomic DNA methylation. On the other hand, a hypermethylation on ALU-M2 sequence up to 30 % was induced when the medium was supplemented with red wine lees at 2000 ppm.

Figure 6 shows the white and red wine lees modulated activity against DEN methylation effect on ALU-M2 sequences. White wine lees reduced the methylation status (demethylation) of DEN at all tested concentrations, while red wine lees increased the methylation pattern (hypermethylation) of DEN up to 130 % at the highest tested concentration (4000 ppm) during the study.

**4. Discussion**

In the present study, , the chemopreventive effects of red wine lees and white wine lees on early stages of hepatocarcinogenesis have been investigated for the first time from a histopatological and methylation point of view. As described above, there is a considerable body of evidence suggesting that wine lees are promising candidate agents for HCC chemoprevention.

The rat model of DEN- induced HCC has been considered one of the best characterized experimental models of this neoplasia. [8,23,24] DEN is a potent hepatocarcinogenic nitrosamine that induce lesion as well as tumors in rodents with marked biochemical, histological and molecular similarity to the progression of human HCC . [24] The main found lesion in this study was cellular alteration focus which has proved to be precursors of human HCC and commonly found following hepatocarcinogen exposure such as nitrosamines. [8,21] These focus may be classified based on the predominant cell type (basophilic, eosinophilic or mixed types) and may be observed grossly as small white nodules in the liver surface. [21] In this study, most of the nodules were of eosinophilic type and more than 95% of them were less than 3 cm in diameter. Sprague-Dawlye rat has shown to be a suitable model to study DEN induced hepatocellular cancer as reporter by others authors. [25] Moreover, in view of the limited treatment options and bad prognosis of this malignancy, this experimental model has been a very useful means for the screening of potential chemopreventive compounds as the best and promising strategy for reducing incidence and mortality of HCC.

Red and white wine has been studied and reviewed extensively as an abundant source of polyphenols (particularly red wine) with chemopreventive activity against carcinogenesis [9] in addition to have many other health benefits. Resveratrol is one of the natural polyphenols found in wine that has demonstrated to prevent hepatocarcinogenesis in rats through suppression of inflammation, oxidative stress and DNA methylation. [12] However, resveratrol is not believed to be the only phytochemical that contribute to the chemopreventive activity of wine since other polyphenols such as quercetin, [26] catechin [27] or gallic acid [27] have alsoshown protective effects . Thus, it has been suggested that other wine extracts might be synergistic with resveratrol resulting in greater effectiveness than the isolated compound. [28] These findings support the need to characterize the wine composition to search new potential chemopreventive compounds. In addition to polyphenols, wine contains a wide range of unexplored components, including wine lees, whose beneficial effects remain unknown. Although it is well known that lees endow wine with organoleptic benefits, the phytochemical composition and anticarcinogenic potential of lees had not been explored before. Thus, the aim of this work was to analyze those wine components in order to clarify if they contribute to the well-known protective activity of the wine in the carcinogenesis and then analyze their potential as natural chemoprevention agents of human HCC.

The phytochemical determination of red wine lees showed that up to 90% of its phenolic content is composed by flavonoids mostly catechin. This phenol, mainly derived from the stems, seeds and skins is often leached out of the grape during the maceration period of winemaking. On the other hand, the flavonoids content in white wine lees is smaller (20% of the total phenolic content) owing to less contact with the skins during winemaking. Both red and white lees contain pyrogallol, GAE and syringic acid but white wine lees contains higher quantities of these components than red wine lees as determined in this study. One of the great differences found between lees is the anthocyanin which represents a key fraction of the phenolic compounds in red wines as other authors have described [29] . Several preclinical and clinical trial studies have shown that phytochemicals such as catechin, GAE and pyrogallol are potential cancer chemopreventive agents [27,30,31] Catechin, in addition to other flavonoids, has been tested and confirmed as a natural aromatase inhibitor in several epidemiological studies most of them in breast cancer [32]. In this study, the lowest doses of red wine lees (1000 ppm) resulted in an increase in body

weight and liver weight, an inhibition of nodular growth and improvement of hepatocellular architecture as compared to the DEN groupHowever, no significant decrease of hepatic fibrosis was observed with any doses of red wine lees. In contrast, our findings reveal that the lowest and highest doses of white wine lees gave place to a notable reduction of nodular growth, increased the body weight and induced an improvement of hepatocellular architecture and DNA hypomethylation In addition, Masson´s trichrome staining revealed that the degree of collagen deposition (fibrosis) decreased with high and low doses of white lees confirming the histopatological findings. Alterations in DNA methylation is a hallmark of rat hepatocarcinogenesis induced in response to a variety of carcinogenic agents. [33,34] Some bioactive food components such as polyphenols haveshown cancer inhibition effects by reducing DNA hypermethylation of key cancer-causing genes [35] All the findings in this study, suggest that white wine lees could prevent the stages of hepatocarcinogenesis inititiated by DEN at the given concentrations.

White wines are made by the free-running juices without pomace, whichno contact with the grape skins. This is the main reason why the phenolic content of white wines is lower than that of red wines. Anticarcinogenic effects have also been attributed to white wine but in a lesser extent than red wine because the difference in inhibition of aromatase activity. [36] However, when white and red wine lees are compared, it seems that white lees show stronger anticarcinogenic potential than red lees. The high content of pyrogallol, GAE and syringic acid present in white wine lees could be the cause of this behavior. Previous *in vitro* studies [37] had shown the strong anticancer activities of these phenolic compounds. Thus our results demonstrate that white wine lees are the best candidate to be used as anticarcinogenic agent through demethylation pathways. Moreover, red and white wine lees could have a potentially different mechanism of chemopreventive actions on liver carcinogenesis that should be further studied. A challenge for future research would be to analyze underlying mechanisms by which these compounds would exert their beneficial effects.

In conclusion, the present findings shown in this study demonstrate a marked inhibitory effect of white wine lees and a small effect of red wine lees in low doses on rat liver carcinogenesis. Thus, wine lees in a large extend, are promising candidate agents for HCC chemoprevention since its beneficial effects on DEN promoted hepatocarcinogenesis in rats. Future studies which include a higher number of rats are needed to assess the results from the present study. In addition, a future goal should be to analyze in detail the underlying molecular mechanisms, such as the differential expression of major oncogenes, through which wine lees can exert their protective effects and determine the most suitable dose for both wine components. These findings may provide strong support for developing novel preventive and treatment strategies for HCC.

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**Legend figures**

Figure 1.- Experimental design for the *in vivo* white and red wine lees experiments.

Figure 2. Red wine lees study. Macroscopic examination (left) and histopathological study (right) at the end of the study. (A) Negative control group showed no nodules in the surface and the typical hepatic lobular architecture. (C) Group 1, (D) group 2, (E) group 3 showed similar liver appearance and architecture as negative control. (B) Group DEN presented multiple small white-yellow nodules in the liver surface and loss of the normal architecture with enlarged hyperchormatic nuclei and mitosis. Rats which received (F) DEN+ 4000 ppm of red lees did not show any effect. However, rats which received DEN + 2000 ppm of red lees (G) and 1000 ppm (H) significantly underwent a decreased of liver nodule growth and improved the hepatic architecture. H&E.

Figure 3. White wine lees study. Macroscopic examination (left) and histopathological study (right) of the livers at the end of the study. A) Negative control group showed the typical lobular architecture of the liver. C) Group 1, D) group 2, E) group 3 showed similar liver architecture as negative control. B) Group DEN presented multiple small white-yellow nodules in the liver surface. G) No significant differences were observed in the liver architecture in the group 2+DEN. F,H) Groups 1+ DEN and 3+ DEN showed significant improvement of hepatocellular architecture with more regular and less altered hepatocytes. H&E.

Figure 4. Masson´s trichrome staining of representative livers sampled from rats in each group. Negative control mice revealed normal lobular architecture and a normal distribution and amount of collagen. Extensive collagen deposition and pseudolobular formation suggesting liver fibrosis is observed in the liver of the rats treated with DEN. Normal distribution and amount of collagen in livers is observed from rats in group 1, 2 and 3. While no differences in fibrosis are observed between rats with 4000, 2000 or

1000 ppm of red lees +DEN, a minor fibrosis can be observed in groups with 4000 and 1000 ppm of white lees + DEN.

Figure 5. Methylation status of ALU-M2 repetitive sequence in rat liver at different concentrations of white and red lees. DEN: diethylnitrosamine; WL: white lees; RL: red lees. Different letters mean significant differences at $P \leq 0.05$ level.

Figure 6. Modulated activity against DEN methylation effect on ALU-M2 sequences in rat liver by different white and red lees treatments. DEN: diethylnitrosamine; WL: white lees; RL: red lees. Different letters mean significant differences at $P \leq 0.05$ level.

**Author contributions**

ZFB and SGL designed and performed most of the experiments, analyzed the data, and wrote the manuscript. YR and MSF helped to perform the *in vivo* experiments and the histopathological analysis of the tumors. JA helped to perform the *in vivo* and *in vitro* assays. PDT and MDLC performed the phytochemical and HLPC-DAD analyses. AAM conceived the project, designed the experiment and revised the manuscript. All authors read and approved the final manuscript. MDLC revised the final manuscript.

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**Conflict of Interest Statement**

All authors declare no financial/commercial conflicts of interest.

451 Tables

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453 Table 1.- Phenolic and anthocyanic compounds identified in red and white wine lees by HPLC-DAD.

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| Compound | Red wine lees (ppm) | White wine lees (ppm) |
|---|---|---|
| Pyrogallol | 7,635 | 12,132 |
| Gallic acid | 8,543 | 9,054 |
| Hydroxymetilfurfural | nq | nq |
| Pyrocatechol | nq | nq |
| Protocatechuic acid | nq | nq |
| Hydroxybenzoic acid | nq | nq |
| Catechin | 91,946 | nq |
| Vanillic acid | nq | nq |
| Guaiacol | nq | nq |
| Vanillin | nq | nq |
| Syringic acid | 2,753 | 9,625 |
| Acetovanillone | nq | nq |
| Coumaric acid | nq | nq |
| Ferullic acid | nq | nq |
| Coniferaldehyde | nq | nq |
| Sinapic Acid | nq | nq |
| Sinapaldehyde | nq | nq |
| Total phenols | 104 | 30 |
| Total anthocyanins | 40.200 | 0.552 |

455 nq: detected but not quantified

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Table 2. Initial and final body weight, liver weight and total number of liver nodules found in the different groups of rats. Values are presented as means ± SD. Different letters mean significant differences at $p \leq 0.05$ level.

| Groups | Initial body weight (g) | Final body weight (g) | Liver weight (g) | Total no. of nodules |
|---|---|---|---|---|
| | | **Red Wine Lees** | | |
| **Control** | 298.3±7.5 | 476.0±14.0 **c** | 15.8±1.4 | 0±0 **a** |
| **1** | 350.7±23.1 | 482.7±24.7 **c** | 18.3±0.3 | 0±0 **a** |
| **2** | 354.7±25.8 | 505.3±30.7 **c** | 19.6±1.7 | 0±0 **a** |
| **3** | 349.0±7.0 | 467.0±16.6 **c** | 17.1±0.5 | 0±0 **a** |
| **DEN** | 338.0±10.4 | 324.3±56.4 **a,b** | 8.4±2.2 | 153.3±5.8 **d** |
| **1 + DEN** | - | - | - | - |
| **2 + DEN** | 354.5±13.4 | 268.0±52.3 **a** | 9.0±1.4 | 112.0±11.3 **c** |
| **3 + DEN** | 337.5±10.6 | 365.5±21.9 **b** | 16.5±3.5 | 95.0±7.0 **b** |
| | | **White Wine Lees** | | |
| **Control** | 298.3±7.5 | 476.0±14.0 **c** | 15.8±1.4 | 0±0 **a** |
| **1** | 299.0±20.5 | 477.0±20.3 **c** | 15.4±1.6 | 0±0 **a** |
| **2** | 307.3±21.8 | 477.7±39.5 **c** | 18.4±2.0 | 0±0 **a** |
| **3** | 295.0±12.3 | 442.7±42.0 **b,c** | 15.8±1.4 | 0±0 **a** |
| **DEN** | 338.0±10.4 | 324.3±56.4 **a** | 8.4±2.2 | 153.3±5.8 **b** |
| **1+ DEN** | 302.0±8.2 | 382.7±3.8 **a,b** | 12.5±0.3 | 15.0±11.3 **a** |
| **2 + DEN** | 297.0±5.6 | 333.7±53.7 **a** | 12.8±5.1 | 116.7±57.7 **b** |
| **3 + DEN** | 299.0±9.5 | 361.0±30.2 **a** | 9.6±0.6 | 49.3±79.4 **a** |