The antimutagenic potential of Vanillin and Betulin based on the antimutagenicity of Resveratrol

O potencial antimutagênico da Vanilina e Betulina baseado na antimutagênicidade do Resveratrol

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ABSTRACT
Due to daily exposure to several compounds with mutagenic potential, it is extremely important to find antimutagenic agents, as here proposed to vanillin and betulin found in *Dipteryx alata* plant, evaluated by the *Salmonella* /microsome assay and resveratrol as reference. The antimutagenicity test employs mutagens that are incubated with a test substance to assess the
inhibitory ability of the mutagenicity, interpreted as absent, moderate, or strong when the percentage of inhibition is lower than 25%; between 25 – 40% or greater than 40%, respectively. The assays were carried out in the absence (-S9) and in the presence (+S9) of metabolic activation using conventional mutagens for TA97a, TA98, TA100 and TA102. Resveratrol strongly inhibited all mutagens from -S9 and +S9. Vanillin moderately inhibited the mutagen of TA97a (-S9), and strongly for all strains in +S9. Betulin moderately inhibited mutagen from TA97a (-S9), strongly for TA100 and TA102 (-S9), and all strains in +S9. The obtained results were all concentration dependent. Concluding, this is an unprecedented study in which all mutagens commonly used in the Salmonella/microsome assay were tested to attribute antimutagenic abilities – from moderate to strong - to vanillin and betulin face to resveratrol, a strong antimitagen.

**Keywords:** Ames test, antimutagenicity, chemoprevention, Resveratrol, Vanillin.

**RESUMO**
Devido à exposição diária a diversos compostos com potencial mutagênico, é de extrema importância encontrar agentes antimutagênicos, como aqui proposto para a vanilina e a betulina encontradas na planta *Dipteryx alata*, avaliadas pelo ensaio *Salmonella*/*microssoma* e o resveratrol como referência. O teste de antimutagenicidade emprega mutágenos que são incubados com uma substância teste para avaliar a capacidade inibitória da mutagenicidade, interpretada como ausente, moderada ou forte quando o percentual de inibição é inferior a 25%; entre 25 – 40% ou acima de 40%, respectivamente. Os ensaios foram realizados na ausência (-S9) e na presença (+S9) de ativação metabólica utilizando mutágenos convencionais para TA97a, TA98, TA100 e TA102. O resveratrol inibiu fortemente todos os mutágenos de -S9 e +S9. A vanilina inibiu moderadamente o mutágeno de TA97a (-S9), e fortemente para todas as cepas em +S9. A betulina inibiu moderadamente o mutágeno de TA97a (-S9), fortemente os de TA100 e TA102 (-S9) e todas as cepas em +S9. Os resultados obtidos foram todos dependentes da concentração. Concluindo, este é um estudo inédito em que todos os mutágenos comumente usados no ensaio *Salmonella*/*microssoma* foram testados para atribuir habilidades antimutagênicas – de moderada a forte – à vanilina e betulina frente ao resveratrol, um forte antimitagênico.

**Palavras-chave:** teste de Ames, antimutagenicidade, quimioprevenção, Resveratrol, Vanilina.

**1 INTRODUCTION**

The search for compounds with antimutagenic potential naturally refers to a disease that is feared worldwide, cancer, which deaths in 2020 are estimated at around 10 million (WHO, 2022). Cancer arises from the failure to repair a genetic mutation, that is, from an alteration in the cell’s DNA, which starts to receive wrong instructions for its activities. Changes can occur in special genes, called proto-oncogenes, or in tumour suppressor genes (INCA, 2022).
Daily exposure to various polluting compounds from industry and traffic present in the air, in cosmetics and even in a large part of food – industrialized or not – can carry contaminants with mutagenic potential (Saeidnia and Abdollahi, 2013; Varanda et al., 1997; Vargas et al., 1990). The mutation is the initiating event of cancer so identifying antimutagens in combating or preventing, or chemopreventing mutational events is extremely important (Almeida et al., 1998; Kada; Morita; Inoue, 1978). According to Waters and McCuthan (1990), the term “antimutagen” was first used by Novick and Szilasrd (1952) to describe those agents that have the property of reducing the rate of spontaneous or induced mutations, regardless of the mechanisms involved.

After the initial observation of the antimutagenic effects of certain plants, several compounds have been isolated from plants and tested for their protective action on induced DNA damage (Mendonça et al., 1998). In Brazil, it is important to highlight the rich biodiversity of its biomes (Sano; Ribeiro; Brito, 2004), such as the Cerrado, which has more than 6,000 vascular plants (Scheffer; Ming; Araujo, 2002), still little explored in Brazil, regarding food and medicinal potential.

The species *Dipteryx alata* Vogel (*D. alata*), popularly known as Baru, a tree of the Leguminosae family, is part of the group of native species used by the regional population as a source of family income. It is one of the most promising species for cultivation due to its multiple uses, high rate of seed germination and seedling establishment (Birrell et al., 2005). The plant is classified as a key species in the Cerrado, since its fruit ripens in the dry season and feeds several species of fauna in this region, including cattle (Rahman; Biswas; Kirkham, 2006).

In addition to the three phytochemicals identified by Coelho Kaplan et al. (1966), a further 15 phytochemicals were isolated and identified by Puebla et al. (2010), therefore, a total of 18 substances are known, namely: four triterpenes, lupeol, lupenone; 28-hydroxy-lup-20(29)-en-3-one and betulin; nine flavonoids, 8-O-methylretusin, 7-hydroxy-5,6,4’-trimethoxysoflavone, afrormosin, 7-hydroxy-8,3',4'-trimethoxyisoflavone, 7,3'-dihydroxy-8,4'-dimethoxyisoflavone, odoratin, 7,8,3'-trihydroxy-4'-methoxy isoflavone, 7,8,3'-trihydroxy-6,4'-dimethoxyisoflavone, dipterixin; a chalcone, isoliquiritigenin; an aurone, sulphidethine; and three phenolic compounds, vanillic acid, vanillin and protocatechuic acid.

Among these substances from *D. alata*, protocatechuc acid, vanillic acid, vanillin (Yoshida et al., 2015) and betulin (Yoshida et al., 2016) were assayed to assess mutagenicity using the Ames test. All these phytochemicals have no mutagenicity.
On the other hand, the compound resveratrol, or 3,5,4′-trihydroxy-trans-stilbene, is a polyphenol found mainly in grapes, red wine, peanuts, dark chocolate, and some berries (Abu-Amero; Kondkar; Chalam, 2016; Wang et al., 2017). Food sources and supplements contain both cis and trans isomers of resveratrol, but the form trans is the most frequently encountered (Aggarwal et al., 2004; Soleas; Diamandis; Goldberg, 1997). Resveratrol was first identified in 1940 in white hellebore roots (Timmers et al., 2011) and later in 1963 in roots used by Chinese and Japanese medicine to treat diseases related to the liver, skin, heart, circulation, and lipid metabolism (Catalgol et al., 2012). Resveratrol has health-promoting properties and antioxidant, anti-inflammatory, cardioprotective, antidiabetics, anticancer, chemopreventive and neuroprotective properties (Botti and Irazusta, 2018; Jeong et al., 2014; Joraholmen et al., 2020; Karthika et al., 2022).

So, the objective of this work was to evaluate the antimutagenic potential of vanillin and betulin found in D. alata by the Salmonella/microsome assay, using resveratrol as an antimutagen reference (Langová et al., 2005; Uenob; Nakamura; Miyazawa, 1997).

2 MATERIAL AND METHODS

2.1 MATERIAL

Based on the amount obtained from the isolation of vanillin and betulin from D. alata (Puebla et al., 2010), it was decided to purchase this compound commercially from the Sigma Chemical Co.® laboratory (St. Louis, MO, USA). Resveratrol (98.7% purity) was obtained from the compounding pharmacy Phyto Formulas (Sorocaba, SP, Brazil).

2.2 FEASIBILITY TEST

TA98 and TA100 were used as strains for the viability test according to the procedure of Espanha (2014). Stock solutions were prepared for each condition. Spontaneous Control: 900 µL saline + 100 µL bacterial culture; Negative Control: 800 µL saline + 100 µL dimethylsulfoxide (DMSO, Sigma®) + 100 µL bacterial culture; Vanillin, Betulin, Resveratrol: 800 µL saline + 50 µL vanillin or betulin or resveratrol (50 mg / 500 µL DMSO) + 50 µL mutagen (for TA98, 4-nitro-O-phenylenediamine or NPD, 10 µg/ plate: for TA100, Sodium Azide, 1.25 µg/plate) + 100 µL bacterial culture. Bacterial culture with a concentration of 1-2x10⁹ cells/mL was prepared from an inoculum made with three layers of bacterial stock stored at -80°C and nutrient broth at
37°C, 100 rpm/14h. Stock solutions were preincubated at 37°C for 20 minutes. After preincubation, they were homogenized by vortexing, flamed, and poured into a tube with 9 mL of saline, repeating the procedure until dilution $10^{-5}$ (Figure 1).

Figure 1. Serial dilution for viability assay. A (800 μL saline + 50 μL sample + 50 μL mutagen + 100 μL TA strain), B (800 μL saline + 100 μL DMSO + 100 μL TA strain), and C (900 μL saline + 100 μL TA strain) show the contents of each stock solution of samples (Vanillin and Betulin compared to Resveratrol), Negative Control and Spontaneous Control, respectively. Each stock solution was pre-incubated at 37°C for 20 min before serial dilution ($10^{-1}$ to $10^{-5}$). For subsequent platelet assays, dilutions from $10^{-3}$ to $10^{-5}$ were selected.

After serial dilution, the viability test was performed in triplicate. Dilutions of 100 μL were selected for the study ($10^{-3}$ to $10^{-5}$) and pipetted into a new tube containing 1 mL of Top Agar supplemented with Biotin/Histidine solution. It was vortexed, flamed, poured into a plate, and incubated in an oven at 37°C for 48 hours. After that, colonies were counted, and colony-forming units (CFU/mL) were calculated. The percentage of viability for each treatment was compared with the number of colonies in the negative control. Responses were considered cytotoxic when the sample survival percentage was less than 80% of the total observed for the negative control. This test is necessary to determine the concentration to be used in the antimutagenicity test, avoiding obtaining false positive results due to toxicity.
2.3 SALMONELLA TYPHOHIMURIM ANTIMUTAGENICITY ASSAYS

In this work, we adopted the changes in the taxonomy of the previously mentioned Salmonella typhimurium for Salmonella enterica subsp. enterica serovar Typhimurium (str. LT2) (Tindall et al., 2005). The procedure described for the Salmonella/microsome mutagenicity test was used (OECD, 2020), with the difference that for the antimutagenicity test, the positive controls of each strain were incubated with the test substances (vanillin or betulin) to evaluate the degree of colony growth inhibition induced by mutagens. Resveratrol was used as a reference (Aggarwal et al., 2004). Assays performed in triplicate were rigorously subjected to controls:

Positive controls: 500 µL Phosphate Buffer pH = 7.4 + 50 µL mutagen in absence of metabolic activation (-S9): 4-nitro-O-phenylenediamine (NPD), 10 µg/plate for TA98 and TA97a; Sodium Azide, 1.25 µg/plate for TA100; Mitomycin C, 0.5 µg/plate for TA102, or in the presence of metabolic activation (+S9): 2-Aminoanthracene (2-anthramine) for all strains, 1.25 µg/plate + 100 µL of bacterial culture.

Negative Control: 500 µL Phosphate Buffer pH = 7.4 + 100 µL DMSO (-S9) or S9 (+S9, Moltox®) + 100 µL bacterial culture.

Spontaneous Control: 500 µL Phosphate Buffer pH = 7.4 + 100 µL bacterial culture; Tests: 500 µL of Phosphate Buffer pH = 7.4 + 50 µL of mutagen + Vanillin or Betulin or resveratrol (different concentrations of the serial dilution) + 100 µL of bacterial culture.

The antimutagenicity results are expressed by the percentage of inhibition, that is, the ability of the compounds to inhibit the action of the known mutagenic agent, according to the equation below (Tachino et al., 1994; Loh; Er; Chen, 2009):

\[
\text{Inhibition} \text{ (%) } = 100 - \frac{(T-M)}{T} \times 100
\]

Where:

\( T \) is the number of revertant colonies on a plate containing mutagen and sample and \( M \) is the number of revertant colonies on a plate containing only the mutagen alone.

Whenever the answer was inconclusive, a confirmatory experiment was carried out. The absence of an antimutagenic effect is verified when the percentage of inhibition was lower than 25%; the effect will be moderate when the percentage of inhibition is between 25 – 40%, and it
will be a strong antimutagen when the percentage of inhibition is greater than 40% (Lira et al., 2008; Neigi; Jayaprakasha; Jena, 2003).

2.4 STATISTICAL ANALYSIS

Data were analyzed using Origin v. 8.0 (OriginLab Corporation, Northampton, MA, USA) and Salanal (Research Triangle Institute, RTP, North Carolina, USA), adopting the model by Bernstein et al. (1982), where data (revert/plate) were evaluated by analysis of variance (ANOVA), followed by linear agents. Compound concentration was expressed as unit mass/plate.

3 RESULTS

This is an unprecedented study in which all mutagens commonly used in the Salmonella/microsome assay such as 4-nitro-O-phenylenediamine or NPD, sodium azide, mitomycin (in the absence of metabolic activation, -S9), and 2-Aminoanthracene (2-anthramine) (in the presence of metabolic activation, +S9) were tested at once with the phytochemical vanillin and betulin, using resveratrol as a reference.

3.1 VIABILITY ASSAY

Before applying the antimutagenicity test, the assessment of the viability test is mandatory aiming to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death. The viability test must demonstrate a percentage of survival greater than 80% in most concentrations and strains, which was demonstrated in this work using TA98 and TA100 (Table 1).
Table 1. Viability assay with TA98 and TA100 strains.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TA98</th>
<th>TA100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls C</td>
<td>136.6 ± 8.3</td>
<td>85.6 ± 16.2</td>
</tr>
<tr>
<td>Controls Cs</td>
<td>177.6 ± 12.0</td>
<td>100.3 ± 3.2</td>
</tr>
<tr>
<td>Resveratrol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>5 ± 2 (3.6%) *</td>
<td>45.6 ± 6.9 (82.8%)</td>
</tr>
<tr>
<td>C2</td>
<td>5 ± 3 (3.6%) *</td>
<td>76.6 ± 57.5 (111.6%)</td>
</tr>
<tr>
<td>C3</td>
<td>152.6 ± 20 (111.7%)</td>
<td>102.3 ± 29 (83.6%)</td>
</tr>
<tr>
<td>Vanillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>4.3 ± 4.1 (3.1%) *</td>
<td>110.3 ± 26.3 (128.7%)</td>
</tr>
<tr>
<td>C2</td>
<td>4 ± 1 (2.9%) *</td>
<td>157.3 ± 12.8 (183.6%)</td>
</tr>
<tr>
<td>C3</td>
<td>166 ± 6.5 (121.4%)</td>
<td>69.6 ± 18.5 (81.3%)</td>
</tr>
<tr>
<td>Betulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>4.6 ± 29 (3.4%) *</td>
<td>45.6 ± 5.5 (53.3%) *</td>
</tr>
<tr>
<td>C2</td>
<td>1.33 ± 1.1 (0.9%) *</td>
<td>76.6 ± 10.40 (89.4%)</td>
</tr>
<tr>
<td>C3</td>
<td>166 ± 14 (121.4%)</td>
<td>102.3 ± 6.8 (119.4%)</td>
</tr>
</tbody>
</table>

Legend: C-, negative control. Cs, spontaneous control. C1=0.0005 (5x10^-5); C2=0.00005 (5x10^-5); C3=0.000005 (5x10^-5). *, toxic values.
Source: Self elaboration.

Taking the TA98 and TA100 together, notice that values > 80% did occur at 0.00005 (5x10^-5) dilution (C3), selected for further experiments. The TA100 strain showed to be more sensitive than TA98 to tested compounds.

3.2 ANTIMUTAGENICITY ASSAY (-S9)

The antimutagenicity assay was first performed without metabolic activation (-S9) (Table 2).

Table 2. Antimutagenicity assay in the absence of metabolic activation (-S9), at 5x10^-5

<table>
<thead>
<tr>
<th>Strains</th>
<th>Controls</th>
<th>Mutagenicity inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C+</td>
<td>C-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA 97a</td>
<td>712.6 ± 43.9</td>
<td>82 ± 14</td>
</tr>
<tr>
<td>TA 98</td>
<td>719.3 ± 47.0</td>
<td>29.3 ± 10.2</td>
</tr>
<tr>
<td>TA 100</td>
<td>1240 ± 27.7</td>
<td>131.6 ± 23.4</td>
</tr>
<tr>
<td>TA 102</td>
<td>1204.6 ± 9.4</td>
<td>266.6 ± 90.1</td>
</tr>
</tbody>
</table>

Legend. C+: mutagens in the absence of metabolic activation (-S9) such as 4-nitro-O-phenylenediamine (NPD), 10 µg/plate for TA98 and TA97a; Sodium Azide, 1.25 µg/plate for TA100; Mitomycin C, 0.5 µg/plate for TA102. All mutagens are at 10^-5 dilution. The percentage of inhibition is expressed in parentheses ( ).
Source: Self elaboration.

The absence of an antimutagenic effect is verified when the percentage of inhibition is lower than 25% which is seen in Table 2 for vanillin and betulin against NPD (TA98) and vanillin for sodium azide (TA100) and mitomycin (TA102). The effect is moderate when the percentage...
of inhibition is between 25 – 40 %, as seen in vanillin using TA97a, and betulin using TA97a. The effect is a strong antimutagen when the percentage of inhibition is greater than 40 % which can be seen with betulin using TA100 and TA102. The reference resveratrol showed to be a strong antimutagen in the absence of metabolic activation.

3.3 ANTIMUTAGENICITY ASSAY (+S9)

Antimutagenicity in the presence of metabolic is shown in Table 3. In this set of experiments, 2-anthramine was used as a mutagen for all strains. Notice that vanillin is a moderate antimutagen only against TA102, while betulin is a strong antimutagenic such as resveratrol.

Table 3. Antimutagenicity assay in the presence of metabolic activation (+S9), at 5x10^-5

<table>
<thead>
<tr>
<th>Strains</th>
<th>Controls</th>
<th>Mutagenicity inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C+</td>
<td>C-</td>
</tr>
<tr>
<td>TA 97a</td>
<td>629.3 ± 74.3</td>
<td>82 ± 14</td>
</tr>
<tr>
<td></td>
<td>(79.3 %)</td>
<td>(89.8 %)</td>
</tr>
<tr>
<td>TA 98</td>
<td>810.6 ± 40.2</td>
<td>29.3 ± 10.2</td>
</tr>
<tr>
<td></td>
<td>(66.1 %)</td>
<td>(98.1 %)</td>
</tr>
<tr>
<td>TA 100</td>
<td>1024 ± 54.9</td>
<td>131.6 ± 23.4</td>
</tr>
<tr>
<td></td>
<td>(81.5 %)</td>
<td>(77.5 %)</td>
</tr>
<tr>
<td>TA 102</td>
<td>217.6 ± 18.7</td>
<td>266.6 ± 90.1</td>
</tr>
<tr>
<td></td>
<td>(59.5 %)</td>
<td>(32.9 %)</td>
</tr>
</tbody>
</table>

Legend. C+: 2-anthramine in the presence of metabolic activation (+S9) (1.25 µg/plate for all strains, following the same serial dilution, 10^-5). The percentage of inhibition is expressed in parentheses ( ). Source: Self elaboration.

4 DISCUSSION

Previously, the Ames test showed that vanillin and betulin are devoid of mutagenicity in the presence (+S9) as in the absence (-S9) of metabolic activation (Yoshida et al., 2015; 2016), becoming strong candidates for the antimutagenicity test. On the other hand, resveratrol has already been tested in the same mutagenicity assay in Salmonella/microsome bacteria (Langová et al, 2005; Uenobe et al., 1997), comet assay and chromosomal aberration showing no genotoxicity in any of these assays (Jeong et al., 2014; Karthika et al., 2022), corroborated by other assays as Allium cepa assay (Botti and Irazusta, 2018). The viability test must demonstrate a percentage of survival greater than 80% in most concentrations and strains (TA98 and TA100) (Espanha, 2014), which was demonstrated in this work.
Resveratrol is a proven antioxidant agent that “quenches” peroxyl radicals (Rimando et al., 2002; Sato et al., 2000), and can prevent oxidative damage to DNA, which plays an important role in the activity of many genotoxic substances (Damianaki et al., 2000; Sgambato et al., 2001). Resveratrol participates in the prevention of carcinogenesis by inhibiting P450, a phase I enzyme (Chang; Chen; Lee, 2001; Gusman; Malonne; Atassi, 2001), and by inducing phase II xenobiotics metabolizing enzymes (Kundu and Surh, 2004; Savouret and Quesne, 2002).

Concerning the Ames test, Uenobe et al. (1997) showed the antimutagenic activity of the compound 3,4',5-trihydroxystilbene (THS) or resveratrol using Salmonella Typhimurium TA1535/pSK1002 against mutagens such as N-Methyl-N -nitro-N-nitrosoguanidine (50.3% of inhibition, -S9), 2-Aminoanthracene (91.1 % of inhibition, +S9), and 3-amino-1,4-dimethyl-5H-pyrido(4,3-b)indole (Trp-P-1) (82.8 % of inhibition, +S9). Langová et al. (2005) showed that resveratrol was antimutagenic against indirect mutagens aflatoxin B1(AFB1) and 2-amino-3-methylimidazo [4,5-f ]quinoline (IQ), but not against the direct mutagen N-nitroso-N-methyl urea (MNU).

In our study, resveratrol showed to be a strong antimutagenic against all mutagens from -S9 and +S9. It means that resveratrol acts directly as antimutagen against 4-nitro-O-phenylenediamine or NPD, sodium azide, mitomycin (in the absence of metabolic activation, -S9), and as an indirect antimutagen against 2-anthramine (in the presence of metabolic activation, +S9).

The compound 4-nitro-O-phenylenediamine is a powerful direct-acting mutagen increasing the risk of neoplasms in humans or animals and affects DNA directly. It is used as a dye in semi-permanent and permanent hair colouring products, as a colourimetric reagent for the determination of ascorbic and dehydroascorbic acids in foods, as a reagent for alpha-keto acids, as a chelating agent for the gas chromatographic determination of selenium in biological materials (PubChem, 2004a).

Sodium azide is not classifiable as a human carcinogen (A4) but as a mutagen. It effectively reverts S. Typhimurium strain TA1530, indicating that it is a base substitution mutagen. It is ineffective on strains which are frameshift mutants. It is highly mutagenic in barley, rice, peas, yeast, and Chinese hamster V79 cells. However, azide does not produce chromosome breaks in barley or Vicia. Sodium azide is a potent mutagen of salmon sperm DNA in an acidic environment. Heritable translocation noted in insects after oral mutation dose 100 mg/L. Sodium
Azide induced a high frequency of mutations in barley seeds. Sodium azide does not induce somatic crossing over and chromosome breaks in soybeans. It is used in organic synthesis, and the preparation of hydrazoic acid, lead azide, and pure sodium. Other uses include the differential selection of bacteria, automatic blood counters, and as a preservative for laboratory reagents. It is also a propellant for inflating automotive safety bags. Agricultural uses include nematocide, herbicide, and fruit rot control (PubChem, 2004b).

Mitomycin is used as an alkylating agent in cancer chemotherapy. The Human Health Assessment Group in the Environmental Protection Agency's Office of Health and Environmental Assessment has evaluated mitomycin C for carcinogenicity, as group B2, which is based on sufficient evidence in animals. No data are available on humans. As a group B2 chemical, mitomycin C is considered probably carcinogenic in humans (PubChem, 2004c).

The mutagen 2-anthramine is produced from the incomplete combustion of organic substances, it is found in synthetic fuel, and used for biochemical research and organic synthesis. Evidence for carcinogenicity is from studies using animals. In mice, subcutaneous (sc) injections induced tumours in the liver; in rats, topical administration induced local tumours and tumours in the breast, sc injections induced skin tumours; in hamsters, topical administration induced local tumours. These vias of administration pose 2-anthramine as an indirect mutagenic compound needing the presence of microsomal enzymes (PubChem, 2004d).

As these mutagens have been used in several human activities exposure to them is not impossible for an event to occur. Natural occurring dietary antimutagens as those found in foods such as fruits and vegetables could counteract the deleterious effects of these mutagens (Bode and Dong, 2009). The mechanism by which resveratrol acts as antimutagen needs to be clear. Among the common mechanisms (antimutagens with antioxidant potency, interaction with mutagen, antimutagen as blocking agents, multifunctionally acting antimutagens, desmutagenesis, and bio-anti mutagenesis) (AbdelHakem and Abdelhafez, 2021), antimutagenicity of resveratrol would be related with antioxidant potency, since the removal of reactive molecules is considered an important strategy in the process of anti-mutagenesis before these molecules react with DNA (Tian et al., 2012). Another mechanism which resveratrol showed to be able is related to the direct chemical interaction between a mutagen and an antimutagenic (AbdelHakem and Abdelhafez, 2021), before it induces DNA damage, as shown against 4-nitro-O-phenylenediamine, mainly.
In the absence of metabolic activation (-S9), vanillin, a phenolic compound, was able to inhibit moderately NPD using TA97a (37 % of inhibition), but not against NPD using TA98 (16 % of inhibition). Both bacterial strains showed dependency on histidine with the presence of rfa mutation, uvrB deletion, and plasmid pKM101. The uvr mutation causes a deficiency in the DNA excision repair system. The rfa mutation causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria, thus increasing their permeability to large molecules. Besides, the standard Ames tester strains of S. Typhimurium contain independently isolated uvrB-bio-gal deletions and rfa mutations, thus most likely they are different (Jurado; Alejandre-Durán; Pueyo, 1993). These two mutations have greatly enhanced the mutagen sensitivity of the Salmonella Ames test (Maron and Ames, 1983). Another difference between TA97a and TA98 is related to the DNA target hisD6610 and hisD3052 for TA97a and for TA98 (DeMarini et al., 1992; Hartman et al., 1986), respectively. Although the mutation is frameshift for both strains, the mechanism by which mutation occurs is also different, i.e., for TA97a occurs an addition of +1 cytosine resulting in a run of 6 cytosines (-C-C-C-C-C-C-), while in TA98 occurs a deletion of GC or CG within a common CGCGCGCG hotspot (De Marini et al., 1992; Hartman et al., 1986; Isono and Younno, 1974; Levin; Yamasaki; Ames, 1982; Mortelmans and Zeiger, 2000; Yoshida et al., 2016).

The same antimitagenic activity is shown for the triterpene betulin, which moderately inhibited the NPD mutagen of TA97a, but not using TA98. We believe that the basis of discussion is the same as vanillin. However, betulin strongly inhibited mutagens of TA100 (sodium azide) and TA102 (mitomycin C). The mutagen-susceptible sequence (hotspot) of TA100 is G.C base pairs, while TA102 is A.T base pairs, causing most base pair substitution (Jurado et al., 1993; Levin et al., 1982).

Under metabolic activation, the results showed the antimitagenicity ability of resveratrol, betulin and vanillin, which phytochemicals are in the human diet. These obtained results show the recovery of antimitagenicity of betulin and vanillin after biotransformation, at least against mutagens such as 2-anthramine. There are explicit recommendations against the use of a unique mutagen for all strains (OECD, 2020), mainly with 2-anthramine. The reasons for that are from the findings (Ayrton; Neville; Ioannides, 1992) about “the microsomal and S9 preparations from control animals could activate 2-amino anthracene, but the efficiency of activation was suppressed by pretreatment of animals with Aroclor 1254. Cytosolic fractions from Aroclor
1254-treated rats could readily activate the pro-mutagen more readily than microsomes. The cytosolic activation of 2-amino anthracene required NADPH and could not be accounted for by possible microsomal contamination. The molybdenum oxygenases appear not to contribute to the cytosolic activation of this pro-mutagen. Authors concluded that (a) the microsomal activation of 2-amino anthracene is catalysed more effectively by enzyme systems other than the P450 I family and (b) an enzyme system capable of activating this carcinogen in vitro is present in the hepatic cytosol.”

Authors argue that "most chemical carcinogens are activated by the cytochrome P-450 families inducible by Aroclor 1254 whereas, in contrast, treatment with this mixture of polychlorinated biphenyls suppresses the S9-mediated mutagenicity of 2-amino anthracene demonstrating that this carcinogen cannot be used to monitor these enzymes. Moreover, since cytosol can readily activate 2-amino anthracene in the absence of microsomal activity, a non-viable S9 system may still give a cytosol-mediated mutagenic response with 2-amino anthracene, but other carcinogens cannot be activated because of the absence of microsomal activity, thus giving rise to false negatives (Ayrton et al., 1992)." We point out that the use of 2-anthramine did not avoid the growth of colonies in all assayed strains (excluding false negative results) and did not interfere with the interpretation of results.

5 CONCLUSIONS

We can conclude that in the absence of metabolic activation (-S9) vanillin and betulin are moderate antimutagens against positive control of TA97a (NPD), but betulin strongly inhibited mutagens of TA100 (sodium azide) and TA102 (mitomycin C), while resveratrol inhibited all mutagens. In the presence of metabolic activation (+S9) all tested compounds inhibited the mutagenicity of 2-anthramine. Resveratrol fulfilled its role of reference, proving to be a good antimutagenicity marker in the presence (+S9) and absence (-S9) of metabolic activation.
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