



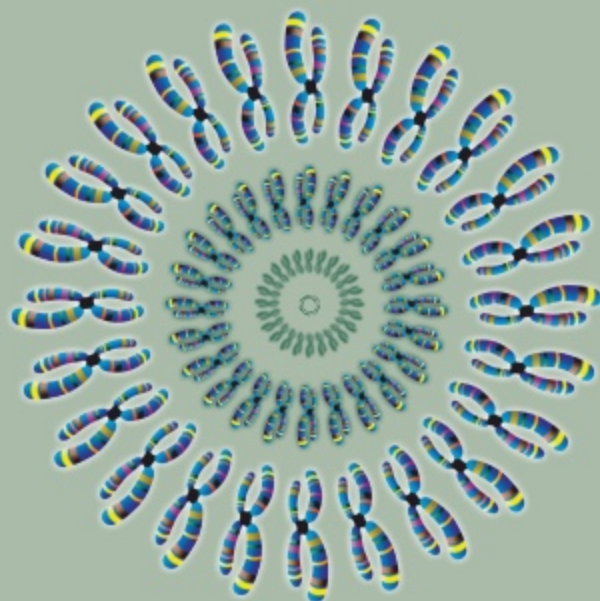
ALEXANDRU IOAN CUZA UNIVERSITY OF IASI



JOURNAL OF EXPERIMENTAL AND MOLECULAR BIOLOGY

Volume 27 Number 3 (2026)

ISSN 2601 – 6974



Alexandru Ioan Cuza University Publishing House

„ALEXANDRU IOAN CUZA” UNIVERSITY OF IAȘI

**JOURNAL OF EXPERIMENTAL
AND
MOLECULAR BIOLOGY**

TOME XXVII, Number 3

2026

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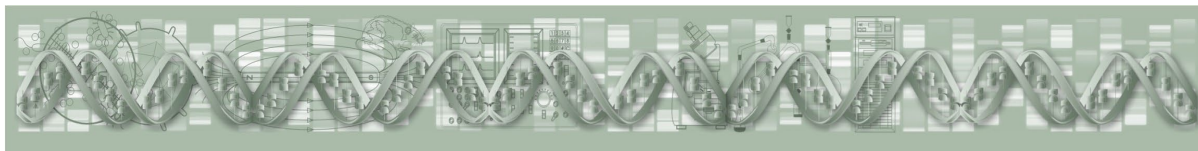
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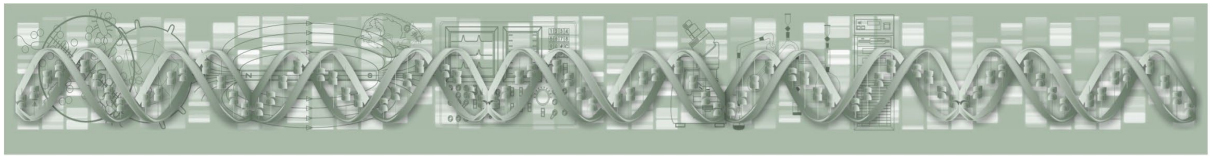
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SENOLYTIC EFFECTS OF BETAINE AMELIORATES DNA DAMAGES AND PHOTOAGEING IN FIBROBLAST CELLS – AN INVITRO STUDY

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Abstract

Ageing is the sequential deterioration of biological functions indispensable for persistence and reproduction. When deterioration reigns above synthesis, the organism ages; UV-induced photoageing is one of the causes of DNA damages leading to premature ageing and there is increased demand for use of phytochemicals as protective agents as senescence inhibitors. Several phytochemicals are being under active research for senolytic effects. In the present study, we focussed to identify the senolytic effects of betaine in UV induced experimental ageing models. L929 cells were subjected to UV treatment to mimic photoageing and the cells were treated with varying concentrations of betaine from 6.25 to 100 µg.mL⁻¹. The impact of betaine on UV exposed cells was determined by reactive oxygen species (ROS) generation using 2'-7'-dichlorodihydrofluorescein diacetate (DCFDA) staining. Comet assay and galactosidase staining was done to analyse the extent of DNA damages and senescence respectively. Betaine-associated protection against UV photoaged L929 cells was evident with concomitant increase in cell viability and significant restoration of cellular morphology. With UV exposure, ROS generation was found to be increased and was reduced in betaine co-treated groups. Betaine significantly reduced UV induced DNA damage as evident from decreased tail length in cotreated groups compared to the increased tail length in UV irradiated groups. X gal staining confirmed the senolytic effects of betaine. The experimental results validate that betaine can curb both oxidative and DNA damages triggered by UV rays proving the potential anti-ageing properties as well as its prospective uses in therapeutic and cosmetic settings.

Keywords: Ultraviolet rays, Photoageing, Betaine, Reactive oxygen species, DNA damage, Comet Assay

Introduction

A combination of unique clinical, histopathological and functional cutaneous changes brought on by prolonged sun exposure is known as photoageing (Berneburg et al 2000). The production of reactive oxygen species (ROS) and genetic alterations are the main harmful consequences of UV radiation on skin. As one of the many forms of solar radiation, UVA often causes the skin to become over exposed to ROS, which can change the structure and functions of genes and proteins. Deep wrinkles, skin laxity and hyperpigmentation all of which are indicative of



photoaged skin are the end result of this process, which also causes the dermis to lose some of its collagen and elastic fibers (Gu et al. 2020).

UV radiation triggers the skin to produce excessive ROS, thereby activating the NF-KB and MAPK signaling pathways (Chen et al. 2016). These two pathways aid in the activation of NF-KB and activator protein-1 [AP-1], which in turn controls the transcription of MMPs, with a focus on upregulating MMP1, MMP3 and MMP9 (Fisher et al. 1996). This mechanism causes an increase in TNF α and MMP expression, which accelerates the ageing process of the skin and degrades the extracellular matrix. The cGas-STING pathway and NF-KB are activated in a similar way by UV radiation, leading to cytoplasmic DNA induction and consequent skin photodamage (Skopelja-Gardner et al. 2020). To conclude, the ageing process is accelerated by UV radiation-induced DNA damage, genomic instability, oxidative stress and extracellular matrix degradation (Tang et al. 2024).

In accordance with recent findings, it is reported that natural products and phytochemicals are preferred for longevity and anti-ageing effects over pharmacological agents but need further scientific studies to identify potent senolytic compounds (Huang et al. 2016). Scientific research on these compounds has expanded over the past decade. Phytochemicals, found in plants, play a crucial role in growth and development (Süntar and Yakıncı 2020), and have been shown due to their antioxidant and anti-inflammatory properties (Zhao et al. 2018, Abruzzo 2020). Trimethylglycine or betaine is a stable nontoxic natural chemical found in plants, animals and microorganisms; it is one of the natural products. Although betaine was originally discovered in beets (*Beta vulgaris*) in the 19th century presence of Betaine is already reported in other plants also (Craig 2004, Cholewa et al. 2018). Recent studies suggest that betaine supplementation may improve endurance, reduce fatigue and enhance muscle power (Arumugam et al. 2020). It supports liver function, protects against liver damage and has anti-inflammatory and antioxidant properties (Ueland 2011, Heidari et al. 2018). Reports suggest the role of betaine in LKB1-AMPK activation inducing autophagy in human epidermal keratinocyte thereby inhibiting UV mediated senescence (Kim et al. 2018). Also betaine protects testicular and ovarian cells from hyperglycemia induced senescence which helps in the management of infertility (Mohammadi et al. 2024). Eventhough most of the studies has confirmed the antioxidant and cytoprotective effects of Trimethylglycine, the senolytic effects and anti photoageing affects needs to be studied in details and in this context the present study investigates the cytoprotective and genotoxicity of betaine with emphasis on the ability of betaine to reduce UV-induced DNA damage in fibroblast cells.

Materials and Methods

Chemicals

Betaine was sourced from Sigma-Aldrich (Cas No: 107-47-3), while all other chemicals were acquired from Invitrogen, Sigma-Aldrich, and HiMedia.

Cell lines

The murine fibroblast cells, L929, were acquired from National Centre for cell science (NCCS) in Pune, India and it cultured in Dulbecco's modified Eagle's medium (DMEM) from Sigma-Aldrich, USA. The medium is enriched with FBS, penicillin, and streptomycin. They were trypsinized using 0.25% and 0.5mM EDTA, then sub-culturing. The cells were then seeded in a 96-well tissue culture plate and maintained at 37°C. The process involved rinsing with PBS, adding trypsin, collecting cells and seeding cells at 5x10³ cells per well followed by incubating plate in a humidified 5% CO₂ incubator.

Induction of in vitro UV toxicity and betaine co-administration

L929 cells were exposed to UV light for 30 minutes as per the protocol of Sunil Kumar et al (Sunilkumar et al. 2024); the media was removed and replaced with complete medium and varying concentration of betaine. The cells were then incubated at 37°C, 5% CO₂ for 24 hours.

SRB assay for cell fixation and staining

SRB assay was done as per protocol mentioned earlier (Nikhitha et al. 2021). For cell fixation and staining, 10% TCA was added to a well plate and incubated at 4°C for 1 hour. The plates were then washed, dried, and then 0.057% SRB solution was added and incubated for 30 minutes to stain the cells. The SRB was removed and rinsed with 1% acetic acid, followed by addition of 200 µL Tris base solution. The plates were shaken for 5 minutes to solubilize the protein-bound dye, and OD was measured using a microplate reader at 510nm

Antioxidant assay

Preparation of cell lysate

Cells were treated with Trypsin-EDTA solution, collected, and centrifuged. The supernatant was discarded, and the pellet was suspended in lysis buffer (0.1M Tris, 0.2M EDTA, 2M NaCl and 0.5% Triton)(Padmanabhan et al. 2011). The samples were incubated at 4°C for 20 minutes, and the resulting cell lysate was used for study.

Estimation of SOD

SOD assay was performed as per the protocol of Mishra and Fridovich (Mishra and Fridovich 1977). Briefly, in 50 µL of cell lysate, 84 µM potassium ferric cyanide, 45 µM methionine, 5.3 mM riboflavin, and, phosphate buffer (pH- 7.8) was added. After that, the tubes were incubated for 10 minutes at 25°C, and the absorbance was measured at 600 nm using spectrophotometer.

Estimation of catalase activity

Catalase was analysed as per the protocol of Ogata and Takahara (Ogata and Takahara 1962). The enzyme reaction was initiated with 1.2 ml of 0.01M phosphate buffer (pH 7.0) and 0.5 ml of cell lysate, followed by 0.1 ml of 0.2 mM H₂O₂. The absorbance decrease was measured at 240 nm and the enzyme blank was run simultaneously with 0.1ml distilled water instead of H₂O₂.

Determination of ROS by DCFDA staining method

L929 cells were seeded onto 96-well plates and incubated for 24 hours at 37°C. The cells were then washed with PBS and treated with 100 µM DCF-DA for 1 hour at 37°C. The resulting fluorescence was imaged on an Olympus CKX41 camera connected to an Optika Pro5 camera, and quantified using a Qubit™ 3 Fluorometer. UV-exposed cells were used as a positive control.

Comet assay

Comet assay was carried out according to the method described earlier (Ramachandran and Saraswathi 2017). Cells were cultured in a 6-well plate, treated with a protective concentration of sample, and incubated overnight. They were then trypsinized and washed with fresh media before being used for a comet assay. Microscope slides were precoated with 0.7% normal melting agarose and stored at 4°C. After each step, slides were incubated at 4°C for 10 minutes to allow agarose to set. Slides were then placed in a cold lysing solution containing 12.5 mM NaCl, 10mM Na₂-EDTA, 10 mM tris base pH 10, and 1% SDS. After lysis, slides were placed in an electrophoresis buffer for 20 minutes to allow DNA unwinding. Electrophoresis was conducted in the same buffer by applying an electric current for 20 minutes. Slides were washed in neutralization buffer three times for 5 minutes, dried, and stained with 50 µl Ethidium Bromide. The slides were photographed using an inverted Epifluorescent microscope and scored using Tritex comet scoring software.

X-gal assay

X-gal assay to determine the extent of senescence was done according to protocol of Sunilkumar et al (Sunilkumar et al. 2024). After washing the cells twice in phosphate buffered saline (PBS), they were fixed for three to five minutes at room temperature using a solution of

2% formaldehyde and 0.2% glutaraldehyde in PBS. After washing the fixed cells in PBS twice, 1 mg/mL X-gal was added to the solution along with 2 mM MgCl₂, 150 mM NaCl, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 40 mM citric acid, and sodium phosphate buffer at pH 6.0. The cells were stained for overnight at 37°C and dried after being washed twice with water. Subsequently, the cells were examined using an Olympus CKX41 inverted phase contrast tissue culture microscope equipped with an Optika Pro5 CCD camera. The microscopic observations were captured as photographs.

Results

Identification of novel senolytic compounds can in turn prevent premature ageing and associated degenerative diseases and our study was focused on assessing the effect of betaine on experimental photoageing induced by UV radiation in fibroblast cells *in vitro*.

In Vitro Cytoprotective Effect Of Betaine On Cultured L929 Cells Exposed To UV Using Srb Assay. Using the SRB assay, betaine's protective effects on L929 cells were evaluated after they were subjected to UV radiation. Cell viability was significantly reduced to 48.59% by UV radiation exposure alone. Nevertheless, the viability of the cells significantly increased to 85.87% when they were treated with betaine and UV simultaneously. According to this observation, betaine improved cell viability under UV exposure conditions by 37%, pointing to possible cytoprotective qualities of the substance (Figure 1A). Furthermore, an epi-fluorescent microscope (Olympus CKX41) fitted with a Pro5 CCD camera was used to observe morphological alterations brought about by different concentrations of betaine, as shown in the Figure 1B. Here after the protective concentration of 100µg/mL of betaine was further used for all the other studies.

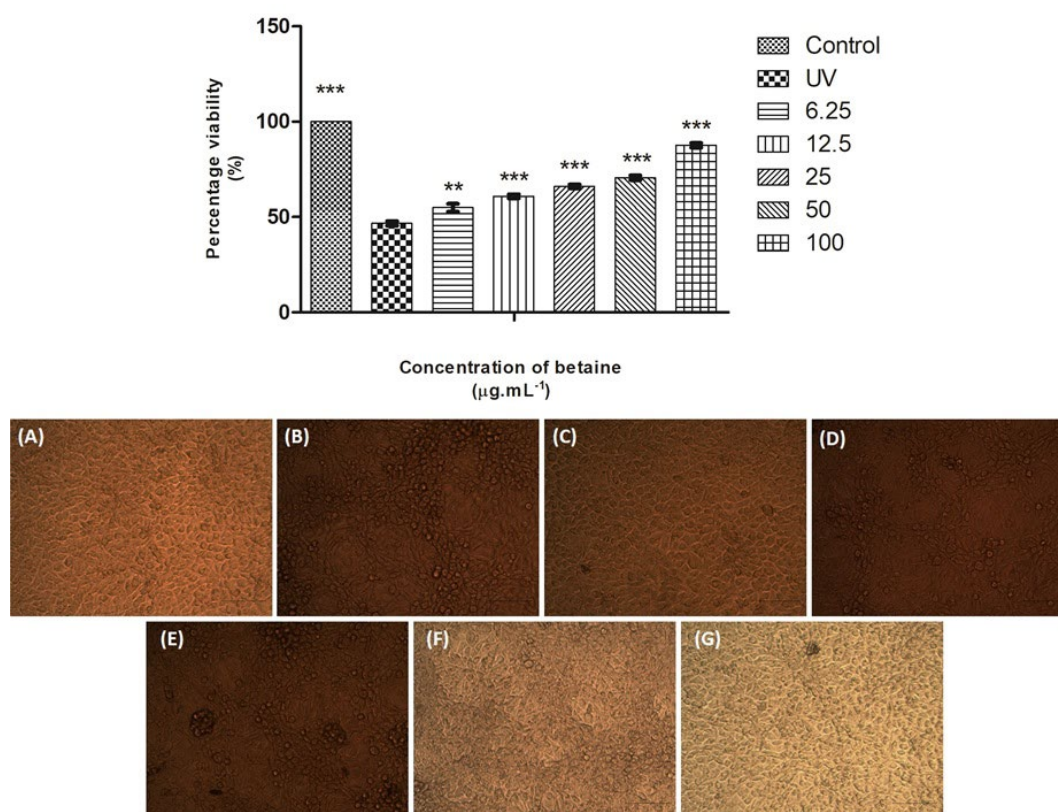


Figure1. *In vitro* cytoprotective effect of betaine on L929 cells exposed with UV - (1A) Graphical representation depicts the *in vitro* cytoprotective effect of betaine in terms of percentage viability (%) on L929 cells exposed with UV, (1B) - Photomicrographs depicting the effect of betaine co-administration on cultured L929 cells exposed to UV (A) Untreated

control cells (B) UV exposed cells and UV exposed cells co-administrated with varied concentration of betaine (C) 6.25 (D) 12.5 (E) 25 (F) 50 and (G) 100 $\mu\text{g/mL}$

Effect of betaine co-administration on antioxidant markers

SOD activity

Research has indicated that the consumption of betaine supplements has the potential to enhance the activity of Superoxide Dismutase (SOD), an essential enzyme within the antioxidant defence system and on the contrary UV irradiation is reported to deplete the cellular antioxidant mechanisms. Superoxide radicals are extremely reactive molecules that have the potential to cause oxidative stress and cell damage. SOD aids in their neutralisation as can be seen from the results, L929 cell lines exposed to UV radiation had much lower SOD activity (0.834 Enzyme units) than untreated control cells (1.539 Enzyme units). When betaine was co-administered with UV-exposed cells, however, SOD activity was noticeably higher (1.44 enzyme units), indicating that betaine had increased cellular antioxidant activity (Figure 2A).

Estimation of Catalase

L929 cells exposed to UV light both alone and in conjunction with betaine had their catalase activity measured. When compared to untreated control cells, the results show that UV exposure significantly reduced catalase activity. On the other hand, catalase activity was significantly elevated when betaine was added in addition to UV exposure (Figure 2B).

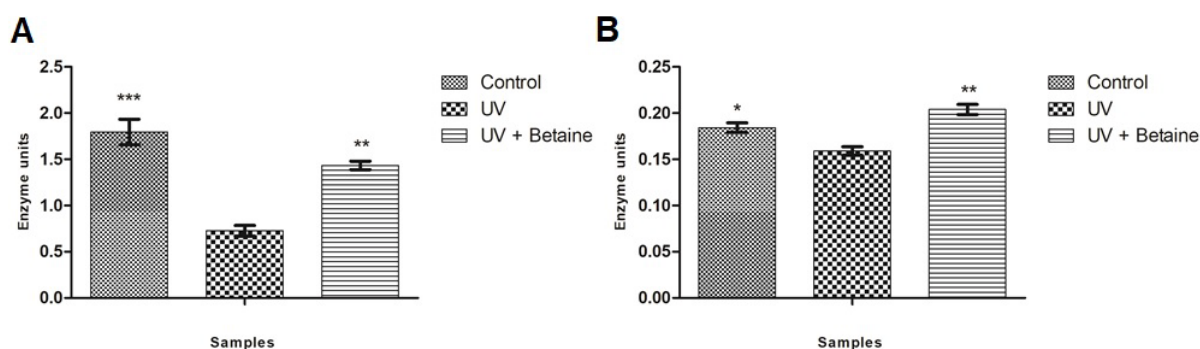


Figure 2. A. Graphical representation depicting the A. SOD level B. Catalase level in terms of enzyme units on the betaine co-administration

Determination of ros generation in uv exposed l929 cells co-treated with betaine

The effects of betaine on intracellular reactive oxygen species (ROS) levels and the formation of ROS in cells exposed to UV light were investigated through experiments. The amount of ROS was measured and expressed as an intensity of fluorescence (AU). The maximum fluorescence intensity of the UV-exposed cells was 37023.02 AU, suggesting a strong generation of reactive oxygen species. Nevertheless, there was a noticeable drop in fluorescence intensity—which registered at 10187.14 AU—when cells were exposed to both UV light and betaine. This decrease in fluorescence intensity points to a decrease in ROS generation in betaine-treated cells, highlighting betaine's potential as a preventive measure against ROS-induced damage initiated by UV-induced cytotoxicity (Figures 3A, 3B).

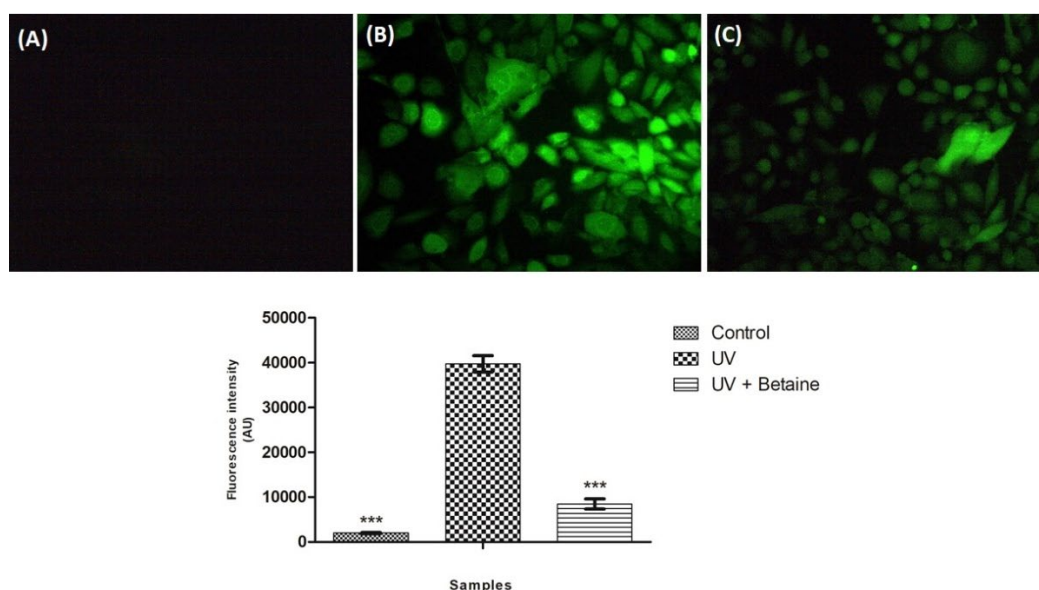


Figure 3. A. Determination of ROS generation by DCFDA staining method: (A) Untreated control cells (B) UV exposed cells (C) UV exposed cells co-treated with betaine; **B.** Graphical representation depicting the generation of ROS on the betaine co-treatment on UV exposed L929 cells

Comet assay

Results showed UV-exposed cells had longer comet tails, indicating more DNA damage which is in accordance with previous findings (Patton et al. 1999).

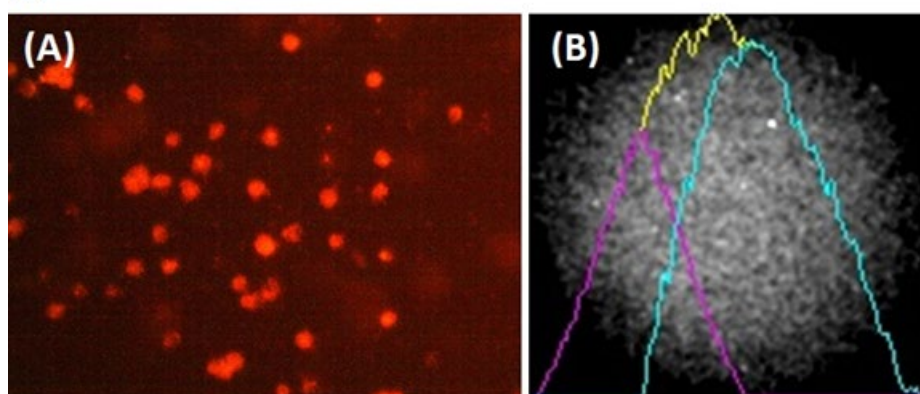
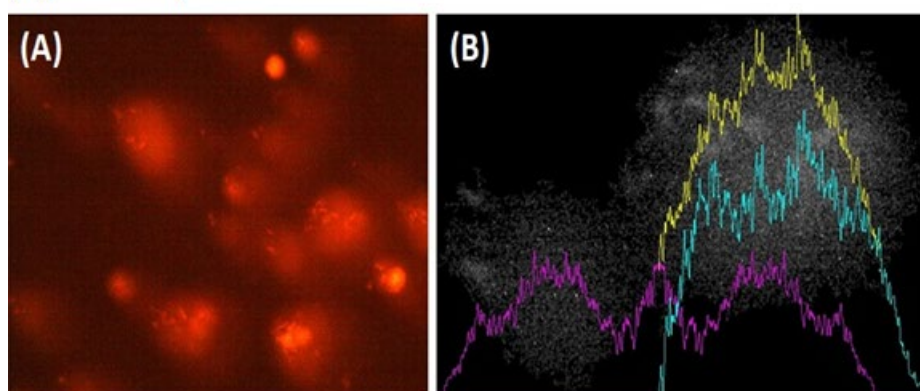
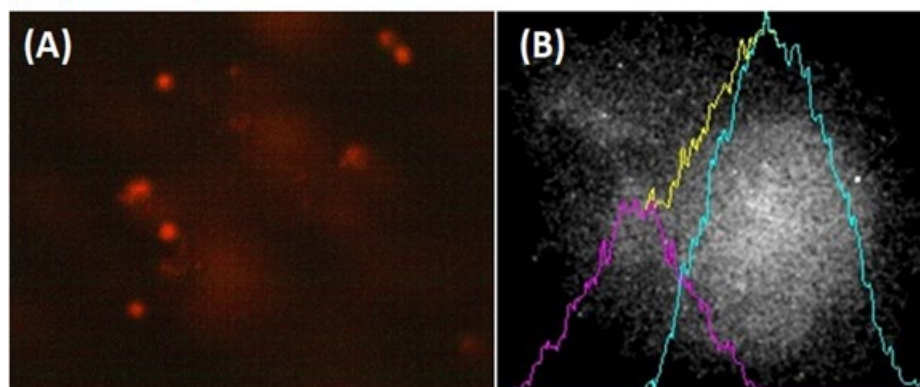
(i) Untreated control cells**(ii) UV exposed cells****(iii) UV exposed cells co-administrated with betaine**

Figure 4. Comet Assay for the determination of genotoxicity: **(i)** untreated control **(A)** representative fluorescent image **(B)** representative scored image of a selected cell, **(ii)** UV exposed **(A)** representative fluorescent image: **(B)** representative scored image of a selected cell, **(iii)** UV exposed co-administered with betaine **(A)** representative fluorescent image: **(B)** representative scored image of a selected cell

However, when cells were treated with betaine, metrics like comet length (Figure 5A), tail length (Figure 5B), and olive moment (Figure 5C) were decreased. The results suggest that betaine provides a positive protective effect against UV-induced DNA damage (Figure 4).

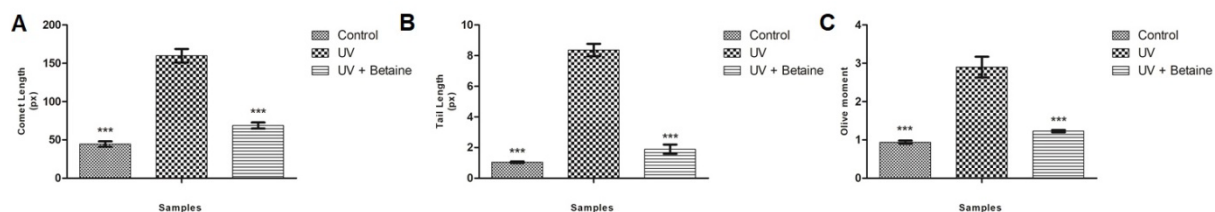


Figure 5. Graphical representation illustrating **A:** comet length, **B:** tail length, **C:** Olive moment

X-gal staining

Cellular senescence is indicated by Senescence-Associated β -galactosidase (SA- β -gal), which is usually measured at pH 6. Lysosomal compartments and other low pH conditions are where it exhibits its highest action. Using a chromogenic substrate like as X-gal, researchers assess SA- β -gal activity using in situ staining techniques. Cultured L929 cells exhibit a blue-stained senescent phenotype upon exposure to UV light, suggesting that UV exposure triggers senescence (figure 6). Nevertheless, the number of these blue-stained, senescent cells drops when betaine is also given UV radiation, indicating that betaine may have a preventive impact on UV-induced senescence in L929 cells (Figure 6).

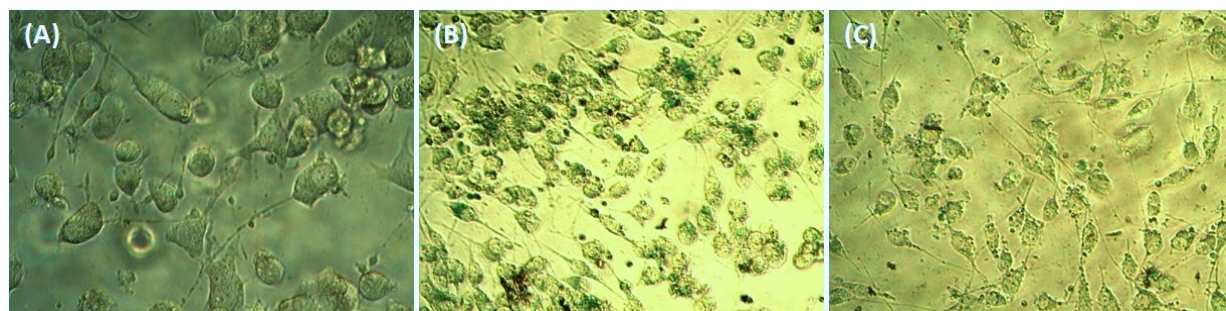


Figure 6. X-gal staining: **(A)** untreated control cells, **(B)** L929 cells exposed to UV radiation, **(C)** L929 cells exposed to UV radiation co-administrated with betaine

Discussions

Our study strives to explore the possible senolytic effects of betaine in lowering UV-induced DNA damage and avoiding cellular degradation in L929 cells, which opens pathways for revolutionary therapeutic treatments in degenerative pathologies.

A naturally occurring substance present in microbes, marine life, and plants is called betaine, often referred to as trimethylglycine. In times of osmotic stress, it is an essential osmolyte that aids cells in preserving fluid equilibrium (Zeisel and Da Costa 2009). Along with helping to methylate DNA and synthesise neurotransmitters (Lever et al. 2005), it also serves as a methyl donor in the methylation cycle. Homocysteine levels are a risk factor for cardiovascular illnesses, and betaine may help lower them (Arumugam et al. 2020). Moreover, by promoting cholesterol metabolism and lessening hepatic steatosis, betaine may benefit liver health. Supplementary research on betaine is warranted due to its various physiological roles and possible health advantages but least studied for its potential effects on photoageing and associated changes.

The L929 cell line was chosen for its anti-ageing properties. The cells were exposed to different concentrations of betaine and UV radiation to assess its protective effects. Treatment with 100 μ g/ml of betaine significantly improved cell viability by 37%. However, UV radiation without betaine treatment reduced viability by 48.59%, accompanied by visible cell morphology changes, suggesting potential apoptosis or cell death.

The study found that endogenous antioxidants, including catalase and SOD, play a crucial role in the body's defense against UV radiation's harmful effects on fibroblast cells. The levels of these antioxidants increased significantly in groups co-treated with betaine compared to those exposed solely to UV radiation.

Intraoperative fibroblast pathophysiology is significantly influenced by reactive oxygen species (ROS), which are produced at higher levels when exposed to UV light. Our data unmistakably show that cells exposed to UV light produce more ROS. Nevertheless, as compared to untreated control cells, co-treatment with betaine reduced the production of ROS which can be attributed to the free radical scavenging potential of betaine. Significant cryoprotection and enhanced cell viability resulted from this reduction in ROS production. It was already reported that betaine can attenuate expression of age related NF kappaB activities induced by oxidative stress thereby preventing ageing and inflammatory activities (Go et al. 2005)

Senescence progresses more quickly when DNA damage occurs. We used single cell gel electrophoresis, an alternative name for the comet test, to assess this damage. Utilising Triton Comet scoring software, DNA damage is evaluated by staining with ethidium bromide and analysis. The comet tail's notable extension following UV radiation exposure, which suggests more DNA damage, is proven by our findings. That comet tail length, however, significantly decreased by 50% after betaine was added, indicating that it had a protective effect against UV-induced DNA damage. Recent reports suggest the oral administration of betaine to reduce UVB-induced skin damage promise its use in cosmetic applications (Im et al. 2016).

When fibroblast cells undergo replicative senescence, there is a rise in detectable beta-galactosidase (beta-gal) activity at pH 6.0, as determined by cytochemical techniques. For assessing cellular senescence in both in vitro and in vivo settings, this increase in activity is a reliable indicator. In order to identify senescence and assess the degree to which betaine attenuates UV-induced senescence, we utilised X-gal staining in recent research. What is evident from the findings is that beta-galactosidase activity is significantly elevated in groups exposed to UV light. Simultaneous betaine administration, on the other hand, successfully lowers X-gal production, indicating a reduction in cellular ageing. Betaine is one of crucial metabolite in natural ageing process and it was reported that the target of rapamycin (mTOR) signaling pathway, forkhead box transcription factor (FoxO) and p38-mitogen-activated protein kinase (MAPK) are the three coregulated metabolic pathways in ageing and the anti-ageing drugs have raised the betaine levels consistently (Lan, W et al. 2024). These results confirm the reduction in cellular senescence observed in the betaine-treated groups and highlight the potential anti-ageing effects of betaine. Recent research into senolytics, agents that selectively eliminate senescent cells, aligns with these findings, emphasizing their role in mitigating ageing and related diseases.

Conclusions

The study demonstrates that betaine or Trimethyl glycine which is a plant-derived phytochemical, exhibits significant potential in counteracting UV-induced photo ageing and DNA damages. UV exposure typically reduced cell viability, but treatment with 100 µg/ml of betaine was found to restore cell viability to 85.87% with concomitant improvement in cell morphology. Additionally, betaine enhanced antioxidants like SOD and catalase while reducing cellular ROS generation in contrary to UV alone treated groups. Importantly, the study highlights betaine's ability to slow cellular ageing and preserve DNA integrity through senescence assays. These findings suggest that betaine has promising anti-ageing properties and potential applications in both therapeutic and cosmetic contexts.

Acknowledgements

The authors would like to acknowledge the Management and Staffs of Center for research on molecular and applied sciences, Thiruvananthapuram for their assistance with providing the necessary infrastructure and laboratory facilities

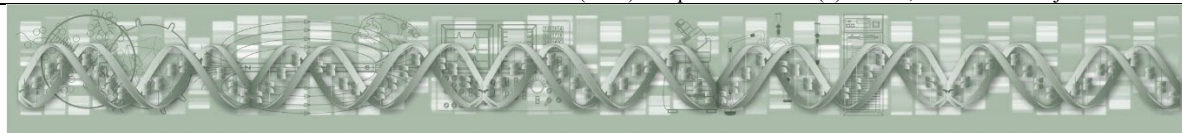
Financial support.

Nil

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EVALUATING THE PERFORMANCE AND NUTRIENT DIGESTIBILITY OF WEST AFRICAN DWARF GOATS FED DIETARY LEVELS OF RICE MILLING WASTES

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Abstract

This study evaluated the performance and nutrient digestibility of West African dwarf goats (WADG) fed dietary levels of rice milling waste (RMW). Twenty-four West African dwarf bucks (average weight 5.49 kg and aged 6–8 months) were used for the study. Four experimental diets were formulated and designated as T1, T2, T3 and T4. T1 was the control and contained 0% of RMW while T2 contained 10%, T3 20% and T4 30% of RMW. The experimental goats were randomly assigned to four the dietary treatments: T1, T2, T3 and T4 in a completely randomized design. The trial lasted 90 days. Nutrient intake, body weight changes, haematology, serum biochemistry and nutrient digestibility were studied. Supplement dry matter intake (DMI) was significantly higher ($p < 0.05$) in T3, while DMI as a percentage of body weight was higher in T1 and T4. Crude protein intake was lower ($p < 0.05$) in T4. Neutral detergent fibre intake was higher ($p < 0.05$) in T4. Acid detergent fibre and acid detergent lignin intakes were high ($p < 0.05$) in T3 and T4. Daily weight gain was highest ($p < 0.05$) in T2. The feed conversion ratio were better ($p < 0.05$) in T1, T2 and T3. Packed cell volume and mean cell haemoglobin concentration were improved ($p < 0.05$) in T1 and T2, while haemoglobin, red blood cell count, and mean cell volume were lower ($p < 0.05$) in T4. Crude protein, neutral detergent fibre and acid detergent lignin digestibility were high ($p < 0.05$) in T1. The study concludes that rice milling waste can be incorporated into the diets of WAD goats at levels up to 30% without adverse effects on performance, or digestibility. Better results in weight gain, feed conversion, haematology and nutrient utilization were observed at 10% inclusion and hence recommended for enhanced goat production.

Keywords: goats, agro-byproducts, nutrient intake, blood profile, growth performance, rice milling waste

Introduction

Goats are among the relatively few ruminant species in the animal kingdom. Goats have a very high potential for productivity, which, with better management, can greatly contribute to economic improvement of the farmer substantially. The subsistence farmer's thinking has not

yet changed from employing goats as a savings asset to managing them as a productive asset, which is regrettably why the goat business has not been as developed and intensified as most other livestock. Goat production in Nigeria is largely extensive, with minimal housing and health management, and animals depend mainly on natural grazing, crop residues, and household wastes with little or no supplementation, in contrast to the USA and Australia where goats are managed under semi-intensive to intensive systems with controlled housing, strategic health programs, and scientifically formulated rations including improved pastures, hay, silage, and concentrate feeds (Silva et al. 2022, Tella and Chineke 2022). According to Adekanbi et al. (2020) feed is the most crucial element in managing goats as productive assets, providing high-quality, fresh, and nutritive feed is essential to profit maximization in goat production. Increasing the productivity of small stock seems to depend more on improved goat nutrition. The least expensive source of nutrients for goats is native rangelands, and for most of the year, the grasslands are unable to offer enough nutrients to goats for increased productivity. High-quality dry season supplements are necessary to boost production because most tropical forage species have low dry matter digestibility (Aregheore 2001), especially when the natural forages are scarce in both quantity and quality during the dry season.

In the tropics, the main constraint to goat production is the supply of affordable, high-quality feedstuffs. Feed accounts for 70–80% of the cost of raising livestock. Due to seasonality, scarcity, and competition between humans and animals for traditional feedstuffs, the ready availability of these feeds is limited. However, non-conventional feed resources (NCFRs), or alternative low-cost feed ingredients, can be used as substitutes to reduce feeding costs (Jiwuba 2020). The wastes from milling rice (rice bran, husk, broken rice), cassava peel, and bambara nut wastes are a few examples of NCFRs. The byproduct known as rice milling waste (RMW) is derived from rice milling industries that process parboiled rice using a mechanism that combines the removal of husk and polishing into a single operation to produce clean grain and rice offal, which contains husk, bran, polishing, and small amounts of broken grains (Dafwang and Shwaremen 1996). Developed nations split this waste into several components and turn them into goods with added value. In Nigeria, however, it is typically used for livestock feeding, improperly discarded in waste heaps causing pollution, or utilized as firewood. Due to its high fibre, and low protein, it has not been well utilized as animal feed despite being readily available (Oyawoye and Nelson 1999). In most parts of Nigeria where rice is produced, one of the most prominent agro-industrial wastes produced in significant quantities is rice milling waste. Nigeria's rice-producing regions are rich in rice milling enterprises, which provide by-products for rice processing (Omotola and Ikechukwu 2006). According to NAERLS and PCU (2004), Nigeria produces an estimated 1,032,993.6 metric tonnes of rice milling by-products annually. The rice industry will in nearest future experience significant decrease in disposal issues and value addition if rice milling wastes are efficiently used as animal feed. However, there is a dearth of information on the utilization of rice milling wastes by goats. The objectives of the study therefore are to evaluate the nutrient intake, body weight changes, haematology, serum biochemistry and apparent nutrient digestibility of goats fed diets containing rice milling wastes. We assume that the inclusion of 0–30% of rice milling waste will have a beneficial effect on nutrient intake, body weight change, haematology, serum biochemistry and apparent nutrient digestibility in West African dwarf goats.

Materials and Methods

Location of the study

The study was carried out at Federal College of Agriculture's sheep and goat unit, Ishiagu, Ivo L.G.A., Ebonyi State, Nigeria. The college is about 3 kilometres from the major town of Ishiagu. The College is located at latitude 5.56° N and longitude 7.31° E, with an average annual

rainfall of 1653 mm, a prevailing temperature of 28.500°C, and a relative humidity of approximately 80%.

Sourcing of the rice milling wastes and formulation of the experimental diets

The rice milling wastes were obtained from a rice processing mill in Eke market Ishiagu, Ivo L. G. A., Ebonyi State, Nigeria. They were further sun-dried in batches and stored for future use. Four experimental diets T1, T2, T3, and T4 were formulated from rice milling waste, brewers dried grain, palm kernel cake, soybean, cassava peels, bone meal, and salt. The rice milling wastes (RMW) were included at the levels of 0%, 10%, 20% and 30% for T1, T2, T3, and T4 respectively as presented in Table 1.

Table 1. Composition of the experimental diets

Ingredient	T1 (0.00%)	T2 (10.00%)	T3 (20.00%)	T4 (30.00%)
Rice milling wastes	0.00	10.00	20.00	30.00
Palm Kernel Cake	40.00	30.00	20.00	10.00
Soya Bean Cake	7.00	7.00	7.00	7.00
Brewer Dried Grain	20.00	20.00	20.00	20.00
Cassava Peel	30.00	30.00	30.00	30.00
Bone meal	2.50	2.50	2.50	2.50
Salt	0.50	0.50	0.50	0.50
Total	100.00	100.00	100.00	100.00

Animal management

Twenty-four West African dwarf bucks weighing about 5.49kg and aged 6 to 8 months were selected from the College herd. For a preliminary period of 14 days, each animal was fed the control diet on 3% of their body weight in the morning (7:00 hour) and 2 kg of wilted *Panicum maximum* that was cut in the morning as basal in the evening (15:00 hour). This was done to increase the appetite of the goats for concentrate feeding. The experimental goats were acclimatized for 14 days before the start of the study, as per the approval and rules of the Animal Ethics Committee of the Federal College of Agriculture, Ishiagu, Ebonyi State, Nigeria.

Nutrient intake and body weight gain

Four groups of six goats each were randomly selected, with each goat serving as a replicate. Using a completely randomised design (CRD), the groups were assigned to the four experimental diets (T1, T2, T3, and T4) at random. Each animal was kept in a separate pen with a concrete floor, adequate air circulation, and drinking and feeding facilities. For 90 days, each animal was fed respective treatment diet on 3% of their body weight in the morning (7:00 hour) and 2 kg of wilted *Panicum maximum* that was cut in the morning as basal in the evening (15:00 hour). Fresh drinkable water was made available regularly. Each animal's voluntary feed intake was calculated daily by subtracting the previous day's feed refusals from the current day's feed supply. The goats' initial live weights were taken using spring balance at the start of the feeding trial and weekly afterwards in the morning before feeding. After the trial, the goats were weighed to determine their final live weight. Other growth performance indexes were computed accordingly.

Digestibility study

Twenty-four, six- to eight-month-old WAD bucks were used in this investigation. Under zero grazing conditions, the goats received supplemental feed at a daily rate of 3% body weight per head on a DM basis. Following their first disinfection, the goats were put in separate metabolism cages equipped with faeces collection equipment. The goats were randomly assigned to the four test diets (T1, T2, T3, and T4) following the adjustment phase in a CRD design. Twice daily, at a rate of 3% of body weight, the goats were fed the diets at 7:00 hour

and 15:00. hour. There was provision of clean wholesome water as free choice. The goats were given experimental diets for 28 days. Data were collected from days 21 to 28. For each buck, the amount of faeces excreted was measured every day for seven days. Over the course of a week, faeces were collected and bulked for each buck. For each animal, 20% of the faecal samples were bulked. After that, the faecal samples were freeze-dried, powdered, and stored for further evaluation.

Proximate analyses

AOAC (2005) procedures were used to analyze all the faecal samples for proximate components. Following AOAC (2005) guidelines, three separate samples of the dietary levels of rice milling were examined for dry matter (DM), crude protein (CP), crude fibre (CF), ash, ether extract (EE), and nitrogen-free extract (NFE). The neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) fractions were determined using Van Soest et al. (1991) methods.

Gross Energy Determination: The formula was used to determine the gross energy.

$T = 5.72Z1 + 9.50Z2 + 4.79Z3 + 4.03Z4 \pm 0.9\%$; according to Nehring and Haelein (1973).

Where;

T = Gross energy;
 Z1 = Crude protein;
 Z2 = Crude fat;
 Z3 = Crude fibre;
 Z4 = Nitrogen free extract.

Experimental design and statistical analyses

The experimental design used was a completely randomized design (CRD). Data obtained were analysed using analysis of variance (ANOVA) as described by SAS (2008). Significant means were separated using the Duncan Multiple New Range Test at $p < 0.05$.

Results

The proximate composition of the experimental diets is presented in Table 2. There are significant ($p < 0.05$) differences for CP, NDF and ADF, while every other parameter measured were not affected ($p > 0.05$). crude protein contents were significantly ($p < 0.05$) high in T1, and T2 in comparison to T4. On the other hand, NDF and ADF followed a similar pattern with T3 and T4 having significantly ($p < 0.05$) higher values than T1. The nutrient intake of West African dwarf goats fed dietary levels of rice milling waste is presented in Table 3. The supplement dry matter intake (SDMI), supplement dry matter intake as percentage of body weight (SDMI % body weight), crude protein intake, neutral detergent fibre intake, acid detergent fibre intake and acid detergent lignin intake showed significant ($p < 0.05$) differences, while forage dry matter intake, total dry matter intake, forage dry matter intake as percentage of body weight, total dry matter intake as percentage of body weight and ash intake remained statistically similar ($p > 0.05$). The dry matter as a percentage of body weight for supplements were significantly ($p < 0.05$) higher in T1 and T4 than for T2 and T3. The CP intake (g/d and g/kg $W^{0.75}$) of the goats fed the T1 and T2 diets had the highest ($p < 0.05$) values (47.19 and 48.12 g/d and 18.00 and 18.27 g/kg $W^{0.75}$ respectively) while T4 had the least ($p > 0.05$) value (36.56 g/d and 14.87 g/kg $W^{0.75}$). The neutral detergent fibre, (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) intakes were significantly ($p < 0.05$) high in T4 in comparison to the other groups. The body weight changes of West African dwarf goats fed dietary levels of rice milling waste is presented in Table 4. The final body weight was significantly ($p < 0.05$) affected with goats in T4 (9.95kg) having the lowest ($p < 0.05$) value in comparison to T1, T2 and T3. Body weight gain/goat was also lower ($p < 0.05$) for animals on diets T4 (2.80 kg) than those obtained in T1 (6.34 kg) T2 (7.33 kg) and T3 (6.12 kg). T4 showed significantly ($p < 0.05$) lower daily weight

gain in comparison with T1, T2 and T3. Daily weight gain was highest for T2 goats (81.44 g/d) and least ($P<0.05$) for T4 goats 31.11 g/d). The feed conversion ratio (FCR) ranged from 3.60 to 9.57 and was significantly ($p<0.05$) high in T4. The haematology of West African dwarf goats fed dietary levels of rice milling waste is presented in Table 5. The packed cell volume (PCV) for T1 and T2 differed ($p<0.05$) significantly from the value of T3 and T4. The haemoglobin (Hb) (g/dl) values obtained in this study showed significant ($p<0.05$) differences among dietary groups, with goats on T4 having significantly ($p<0.05$) lower values than the goats on the other dietary treatments. The red blood cells (RBC) were significantly affected ($p<0.05$) by treatment diets in the present study. The RBC and mean cell volume followed a similar trend as the PCV and Hb. T4 differed ($p<0.05$) significantly with T1, T2 and T3. T4 showed a significantly lower value when compared to the other treatments. Mean cell haemoglobin concentration was significantly ($p<0.05$) high in T1 and T2 when compared to T3 and T4. The serum biochemical indices of West African dwarf goats fed milling waste are presented in Table 6. All the serum biochemical indices examined in this study showed no significant difference ($p>0.05$) across the groups. The apparent nutrient digestibility of West African dwarf goats fed dietary levels of rice milling waste is presented in Table 7. Dry matter digestibility showed a significant ($p<0.05$) difference with T1 and T2 having high ($p<0.05$) DM digestibility in comparison with T3 and T4. CP digestibility was least digested in T4 (47.69%) and better digested in T1 (58.32%), T2 (55.52%) and T3 (56.36%). Crude protein and neutral detergent digestibilities were significantly ($p<0.05$) high in T1 in comparison with T3 and T4, but similar to T2. Acid detergent lignin digestibility showed significantly ($p<0.05$) lower digestibility for T3 and T4 when compared with T1, which was also higher ($p<0.05$) than T2.

Table 2. Chemical composition of the experimental diets

Parameters (%)	T1 (0%)	T2 (10%)	T3 (20%)	T4 (30%)	SEM
Dry Matter	90.51	89.53	91.87	92.05	6.38
Crude protein	16.42 ^a	16.19 ^a	14.97 ^{ab}	12.63 ^b	1.73
Crude fibre	14.56 ^d	16.62 ^c	19.28 ^b	23.76 ^a	1.56
Ether extract	3.87	3.86	4.02	3.73	0.11
Ash	9.76	8.53	9.68	9.05	0.17
Nitrogen free extract	45.90	44.33	43.92	42.88	1.56
Gross energy (Kcal/g)	382.98	387.54	393.18	394.24	12.87
Neutral detergent fibre	36.97 ^b	45.65 ^{ab}	48.35 ^a	51.87 ^a	3.16
Acid detergent fibre	20.78 ^b	23.19 ^{ab}	26.73 ^a	30.09 ^a	2.68
Acid detergent lignin	10.80	13.62	14.70	15.18	1.43

^{a-b} means within the same row with different superscripts are significantly different ($P<0.05$)

Table 3. Nutrient intake of West African dwarf goats fed dietary levels of rice milling waste

Parameters	T1 (0%)	T2 (10%)	T3 (20%)	T4 (30%)	SEM
Dry matter intake (DMI) (g/d)					
Supplement	260.13 ^b	262.41 ^b	278.45 ^a	266.48 ^{ab}	17.23
Forage	30.78	30.44	31.24	31.30	3.15
Total DMI	290.91	292.85	309.69	297.78	13.18
Total DMI (g/kgW ^{0.75})	70.44	70.79	73.82	71.68	3.25
Total DMI % body weight (BW) (%)					
Supplement	3.01 ^a	2.69 ^b	2.61 ^b	2.98 ^a	0.11
Forage	0.36	0.31	0.29	0.35	0.06
Total DMI % BW	3.37	3.00	2.90	3.33	0.12

Total DMI % BW (g/kgW ^{0.75})	2.49	2.28	2.22	2.47	0.08
Crude protein intake (g/d)	47.19 ^a	48.12 ^a	45.52 ^{ab}	36.56 ^b	11.32
Crude protein intake (g/kgW ^{0.75})	18.00 ^a	18.27 ^a	17.52 ^a	14.87 ^b	1.92
Ash intake (g/d)	28.05	25.00	29.43	26.14	2.17
Ash intake (g/kgW ^{0.75})	12.19	11.18	12.64	11.56	1.02
Neutral detergent fibre intake (g/d)	106.25 ^d	133.80 ^c	147.24 ^b	150.16 ^a	9.41
Neutral detergent fibre intake (g/kgW ^{0.75})	33.09 ^d	39.34 ^c	42.27 ^b	42.90 ^a	2.87
Acid detergent fibre intake (g/d)	59.72 ^c	67.97 ^b	81.29 ^a	87.11 ^a	10.74
Acid detergent fibre intake (g/Wkg ^{0.75})	21.48 ^c	23.67 ^b	27.07 ^a	28.51 ^a	3.68
Acid detergent lignin intake (g/d)	31.04 ^c	39.92 ^b	44.70 ^a	43.95 ^a	3.54
Acid detergent lignin intake (g/kgW ^{0.75})	13.15 ^c	15.88 ^b	17.29 ^a	17.07 ^a	0.84

^{a-d} means within the same row with different superscripts are significantly different (P<0.05)

Table 4. Body weight changes of West African dwarf goats fed dietary levels of rice milling waste

Parameters	T1 (0%)	T2 (10%)	T3 (20%)	T4 (30%)	SEM
Initial body weight (kg)	6.31	6.43	7.54	7.15	0.42
Final body weight (kg)	12.65 ^a	13.76 ^a	13.66 ^a	9.95 ^b	0.81
Body weight gain (kg)	6.34 ^a	7.33 ^a	6.12 ^a	2.80 ^b	0.11
Daily weight gain (g/d)	70.44 ^b	81.44 ^a	68.00 ^b	31.11 ^c	3.32
FCR	4.13 ^b	3.60 ^b	4.55 ^b	9.57 ^a	0.85

^{a-c} means within the same row with different superscripts are significantly different (P<0.05)
FCR = Feed conversion ratio.

Table 5. Haematology of West African dwarf goats fed dietary levels of rice milling waste

Parameters	Diets				SEM
	T1 (0%)	T2 (10%)	T3 (20%)	T4 (30%)	
Packed Cell Volume (%)	29.87 ^a	31.76 ^a	27.65 ^b	26.97 ^b	1.76
Haemoglobin (g/dl)	11.48 ^a	11.41 ^a	10.65 ^a	9.08 ^b	0.26
Red blood cell (x10 ¹² /L)	16.72 ^a	15.87 ^a	16.12 ^a	11.74 ^b	0.35
Mean cell volume (fL)	23.17 ^a	19.65 ^a	21.54 ^a	17.04 ^b	0.93
Mean cell haemoglobin conc. (%)	35.22 ^a	35.79 ^a	33.78 ^b	30.37 ^b	0.71
Mean cell haemoglobin (pg)	6.75	5.81	7.99	7.15	1.01
White blood cell counts (x10 ⁹ /L)	8.19	9.05	7.43	8.69	0.54
Lymphocyte (%)	58.90	61.62	57.76	59.24	0.49
Neutrophil (%)	25.84	28.41	28.92	27.32	0.75

^{a-b} means within the same row with different superscripts are significantly different (P<0.05)

Table 6. Serum biochemistry of West African dwarf goats fed dietary levels of rice milling waste

Parameters	Diets				SEM
	T1 (0%)	T2 (10%)	T3 (20%)	T4 (30%)	
Cholesterol (mmol/l)	3.01	2.96	2.83	3.11	0.22
Creatinine (μmol/ l)	70.65	73.87	71.32	72.62	1.64
Urea (mg/dl)	15.56	15.61	14.79	14.33	0.58

Total protein (g/l)	65.87	67.19	66.01	67.35	0.76
Globulin (g/l)	30.86	31.12	30.93	30.63	1.10
Albumin (g/l)	35.01	36.07	35.08	36.72	1.57
Total bilirubin (μmol/l)	0.76	0.43	0.46	0.21	0.11
AST (U/L)	54.86	55.78	53.65	51.98	1.61
ALT (U/L)	19.35	17.82	18.69	21.94	0.11

^{a-b} means within the same row with different superscripts are significantly different (P<0.05)

Table 7. Apparent nutrient digestibility of West African dwarf goats fed dietary levels of rice milling waste

Parameters (%)	Diets				SEM
	T1 (0%)	T2 (10%)	T3 (20%)	T4 (30%)	
Dry matter	78.66 ^a	74.54 ^a	67.56 ^b	68.19 ^b	2.62
Crude protein	64.54 ^a	60.76 ^{ab}	56.76 ^b	57.54 ^b	3.09
Ash	56.73	56.82	58.17	53.49	2.51
Neutral detergent fibre	68.11 ^a	63.67 ^{ab}	58.65 ^b	60.55 ^b	7.71
Acid detergent fibre	43.44	41.07	39.65	38.26	1.87
Acid detergent lignin	47.11 ^a	37.34 ^b	22.71 ^c	22.43 ^c	2.21

^{a-c} means within the same row with different superscripts are significantly different (P<0.05)

Discussions

The CP value of 12.63-16.42% obtained in this study is higher than the 10 to 12% minimum protein requirements for goats as recommended by ARC (1985) and well above the 7.0–8.0 g/100g DM recommended as grave limit, below which intake by ruminants and rumen microbial activities may be negatively affected (Van Soest, 1994). It is important to highlight that the experimental diets' crude protein and energy needs fall within the ranges reported for goats by ARC (1980), NRC (1981), and Norton (1994). The increased supplement DM intake for 20% RMW may be attributed to the greater palatability of the diets. The improved intake obtained in this study can be described by the better supply of both nitrogen and readily available carbohydrates to the rumen microbes which possibly enhanced the microbial growth, the fractional outflow of liquid materials from the rumen, and the rate at which the diets degrade. In an earlier study, Mpairwe et al. (2003) observed that providing supplements with adequate CP to ruminants could enhance DMI, rumen degradation and nutrient flow to the small intestine and culminated in higher animal performance. Across all groups, the dry matter percentages (2.90–3.37%) compared favourably to the 3%, 2.8%, and 2.8%–4.0% percentages recommended daily DM intake (as % BW) requirements for meat type goats in the tropics by Devendra and MCleroy (1982), Akinsoyinu (1985) and Nuru (1985) respectively. The results generally indicated that the animals on the various diets showed positive DM status as evidenced by a generally positive performance of the goats. Protein intake is one of the major determinants of goat performance due to the increased availability of fermentable nitrogen and other nutrients required by rumen microbes as well as the increased chances of some protein escaping the rumen fermentation. The high CP intake of goats fed T1 and T2 could be attributed to the higher CP content of the diets because of the higher protein content of the diet. This is consistent with previous findings by Jiwuba et al. (2023) that a diet high in protein content increases intake. High dietary fibre content may be the cause of the high fibre fraction intake. ADF and ADL-rich diets are predicted to improve resistance to rumen fullness and physical breakdown, which will reduce voluntary intake. Thus, the lower ADF and ADL intake recorded for T1 might be attributed to the high levels of ADF and ADL in the diets which corroborate

the view of Van Soest et al. (1991) who claimed that quantity of fibre fractions might be used to forecast intake. The fact that every goat in the groups gained weight supports Devendra and McIeroy's (1982) findings that a CP of 11% is sufficient for goats to gain weight normally. The highest live weight gain obtained by goats on T1 T2, and T3 may be attributed to the significant CP intake of the goats. The variation observed in the body weight changes of the goats could also be attributed to the variation in the nutrient utilization by each animal from the diets. The goats fed T4 had the highest feed conversion ratio, which is calculated by dividing feed intake by the gain in weight. The lower feed conversion ratio obtained for goats fed T1, T2 and T3 could be attributed to the diet that boosted the multiplication of rumen microbes which must have enhanced the degradability of the nutrients. The composition of packed cell volume, which is employed as a feed toxicity measure, differs among goat breeds (Ahamefule et al., 2005). In the present study, the PCV values ranged between 26.97 – 31.76% and nevertheless fell within the normal physiological range of 22-38% for clinically healthy goats by Radostits et al. (2006), and 21-35% by Daramola et al. (2005) for clinically healthy WAD goats. Aikhuomobhogbe and Orheruata (2006) observed that low PCV results in anaemia which causes a reduced oxygen-carrying capacity of the blood, increased pulse rate and consequently heart failure. Nonetheless, the values found here were within the typical physiological range for clinically healthy goats (8–12 g/dl) (Radostits et al. 2006). Also, the Hb range of 9.08 – 11.48 g/dl obtained in this current study fell within the reference range of 7 – 15g/dl reported by Daramola et al. (2005) for clinically healthy WAD goats. It is possible that the fact that the experimental goats' blood oxygen-carrying capacity was within the reference range for WAD goats suggests that all of the diets provided excellent blood oxygen-carrying capacity. Jiwuba and Kadurumba (2019) support the idea that the high quality of the dietary proteins may be indicated by the Hb concentration of WAD goats on the diets. The RBC values ($11.74 - 16.72 \times 10^6/\mu\text{l}$) recorded for the WAD bucks herein fell within the normal physiological range of $8 - 18 \times 10^6/\mu\text{l}$) reported by Radostits et al. (2006) for clinically healthy goats. Red blood cell characterizes anaemia and other conditions affecting red blood cells (Etim et al. 2013). This result thus shows that the diets supported the good health status of the goats, and hence the animals were not anaemic. The significant and within normal physiological range observed in this study for MCV is an indication that the nutritional quality of the diets were not compromised. The MCV values (17.04 - 23.17 fl) in this study fell within the reference ranges of 16-25 fl by Radostits et al. (2006) respectively for apparently healthy goats, confirmed that the goats were not anaemic. The non-significant effect reported for the serum biochemical parameters may indicate that the diets were not toxic but nourishing since all the parameters were within the normal physiological range for apparently healthy goats reported by Kaneko et al. (1997). Generally, the DM digestibility values were high and ranged between 68.19 and 78.66%. This compared well to goats fed oil palm fronds treated with *Lentinus sajor-caju*, the coefficient of DM digestibility ranged from 68.65 to 78.66% (Hamchara et al. 2018). However, it was lower than the 82.20 to 88.70% found for WAD goats fed fungal-treated rice straw-based diets (Wuanor and Carew 2018) and higher than 54.43 to 62.98% found by Udo et al. (2021) for WAD goats fed varying levels of boiled rubber seed meal-based diets. Differences in the diets fed may account for the differences in the observed results. The higher CP digestibility coefficients reported in T1 and T2 may indicate that dietary proteins were better utilized by the goats fed the respective diets. The crude protein contents of the T1 and T2 diets were positively correlated with fibre fraction digestibilities. This lends credence to the claim made by Fasae et al. (2005) that CF digestibility declines with dietary CP levels. The harmonizing of nutrients, especially protein and energy, should be a key factor in farm animal management in order to maximise nutritional utilisation. Improved digestibility at higher nitrogen levels had previously been linked to low fibre levels (Kayouli et al. 1993). The lignin content of the feed mixtures could be responsible for the discrepancies in digestibility values of fibre fractions reported for the

treatment groups. According to Mertens (1977), changes in cell wall composition including lignin and possibly silica, reduce the possible extent of digestion. Fibre fraction contents in the diets containing rice milling waste were higher and this may have impacted negatively on digestion due to its low microbial colonisation rate in the rumen. It is remarkable to note that the rate of digestion is controlled by chemical entities other than the crystalline or physical nature of the fibre.

Conclusions

It could be concluded that T1, T2 and T3 showed significant improvement in crude protein intake, daily weight gain, feed conversion ratio, packed cell volume, haemoglobin, red blood cells, mean cell volume, mean cell haemoglobin concentration, dry matter digestibility, crude protein and neutral detergent fibre and hence recommended for enhanced WAD goat production. Lastly, higher inclusion levels of rice milling waste above 10% should be discouraged.

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MELATONIN ATTENUATES ALCOHOL-INDUCED OXIDATIVE STRESS AND DUODENAL INJURY IN WISTAR RATS

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Abstract

Long-term alcohol intake can damage the lining of the small intestine by increasing harmful oxidative processes. Melatonin is a naturally produced substance in the body known for its strong protective and antioxidant properties. This study examined whether melatonin could protect the duodenum against alcohol-induced injury in adult rats. Twenty rats were divided into four groups and treated for twenty-eight days with alcohol, melatonin, both substances together, or neither. Changes in body weight, antioxidant activity, lipid damage, and duodenal tissue structure were assessed. Alcohol exposure reduced weight gain, weakened antioxidant defenses, increased lipid damage, and caused marked injury to the duodenal lining. Treatment with melatonin significantly improved weight gain, restored antioxidant balance, reduced tissue damage, and preserved normal duodenal structure. Melatonin alone produced no harmful effects. These findings show that melatonin effectively protects the duodenum from alcohol-induced damage by reducing oxidative stress.

Keywords: Alcohol-induced injury, Antioxidant enzymes, Duodenum, Melatonin, Oxidative stress

Introduction

Alcohol-induced gastrointestinal injury remains a significant global health concern, contributing to chronic mucosal inflammation, epithelial erosion, oxidative stress, and ulcer formation (Chen and Haber 2021). The duodenum, being the first segment of the small intestine exposed to gastric chyme and ingested irritants, is particularly vulnerable to ethanol-induced oxidative damage (Beck 2017). High concentrations of alcohol have been shown to disrupt mucosal integrity through excessive generation of reactive oxygen species (ROS), lipid peroxidation, and impairment of the endogenous antioxidant defense system (Das and Vasudevan 2007). These biochemical disturbances lead to villus blunting, crypt degeneration, inflammatory infiltration, and in severe cases, ulcerative lesions (Akinrinde and Ajibade 2024). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH) constitute the primary enzymatic barrier against oxidative injury in the gastrointestinal tract (Halliwell 2012). Ethanol ingestion depletes these antioxidant systems while simultaneously increasing malondialdehyde (MDA), a key marker of lipid peroxidation (Albano 2006). The resulting redox imbalance plays a critical role in the pathogenesis of alcohol-induced duodenal mucosal injury (Chung et al. 2025). Consequently, research interest has increased in identifying natural or endogenous compounds capable of restoring mucosal protection through antioxidant, anti-inflammatory, or cytoprotective mechanisms.

Melatonin, a neurohormone synthesized by the pineal gland and extensively produced in the gastrointestinal tract, has emerged as a potent mucosal protective agent. Beyond its circadian functions, melatonin exhibits strong free-radical scavenging activity, enhances antioxidant enzyme expression, and stabilizes epithelial tight junctions (Mukhopadhyay et al. 2022). Experimental evidence demonstrates that melatonin attenuates alcohol-induced gastric and intestinal injury by reducing oxidative stress, suppressing pro-inflammatory cytokines, and promoting epithelial regeneration (Kolli et al. 2013, Brzozowska et al. 2014, Karaaslan and Suzen 2015). Its role in improving mucosal microcirculation and preventing ethanol-mediated apoptosis further supports its therapeutic potential in gastrointestinal disorders (de Talamoni et al. 2017).

Despite extensive documentation of melatonin's gastroprotective effects, limited studies have specifically evaluated its protective effect on the duodenum following chronic alcohol exposure, particularly using integrated biochemical and histomorphological endpoints. Understanding this relationship is essential because duodenal injury contributes significantly to malabsorption, dyspepsia, and increased susceptibility to further intestinal pathologies.

Therefore, this study investigates the effects of melatonin on alcohol-induced duodenal injury, assessing changes in body weight, oxidative stress biomarkers (SOD, CAT, GPx, MDA), and histological architecture in Wistar rats after 28 days of exposure. This will provide additional insight into the therapeutic potentials of melatonin in alcohol-related intestinal pathology.

Materials and Methods

Experimental Animals

Twenty (20) adult Wistar rats of both sexes, weighing 160–180 g, were obtained from the Animal House of the Department of Anatomy. The animals were housed under standard laboratory conditions (12 h light/12 h dark cycle, temperature 22–25 °C, humidity 50–60%) and allowed free access to commercial pellet feed and water ad libitum. Animals were acclimatized for 7 days before experimentation. All procedures were conducted according to institutional guidelines for the care and use of laboratory animals and in compliance with international ethical standards for animal research.

Experimental Design

The rats were randomly divided into four groups (n = 5 per group):

- **Group 1 (Control):** Received distilled water only.
- **Group 2 (Alcohol Only):** Received 1 ml of 50% ethanol daily.
- **Group 3 (Melatonin + Alcohol):** Received melatonin (5 mg/kg) + 1 ml of 50% ethanol.

- **Group 4 (Melatonin only):** Received melatonin only (5 mg/kg body weight) orally.

All administrations were given orally once daily for 28 consecutive days using an orogastric cannula.

Body Weight Measurement

Body weights were recorded on Day 0 (initial weight) and Day 28 (final weight) using a digital weighing balance. Weight change for each animal was calculated as:

Weight Gain (g) = Final Body Weight – Initial Body Weight.

Tissue Collection

On Day 29, animals were anaesthetized with ketamine (80 mg/kg). The duodenum was carefully excised.

- A portion was fixed in 10% neutral buffered formalin for histology.
- The remaining portion was immediately rinsed in ice-cold saline, blotted dry, weighed, and homogenized for biochemical analyses.

Preparation of Tissue Homogenate

Duodenal tissues were homogenized in ice-cold phosphate-buffered saline (PBS; pH 7.4) using a Teflon homogenizer to yield a 10% (w/v) homogenate. The homogenate was centrifuged at 3000 rpm for 15 minutes at 4°C, and the supernatant was collected for assay of oxidative stress markers.

Biochemical Analysis

Superoxide Dismutase (SOD) Activity

SOD activity was determined using the method described by Vives-Bauza et al. (2007) based on the inhibition of pyrogallol autoxidation. Results were expressed as units per milligram of protein (U/mg protein).

Catalase (CAT) Activity

CAT activity was measured according to Vives-Bauza et al. (2007) method, which evaluates the rate of decomposition of hydrogen peroxide at 240 nm. Results were expressed in U/mg protein.

Glutathione Peroxidase (GPx) Activity

GPx activity was measured using the method of Vives-Bauza et al. (2007) based on the oxidation of reduced glutathione in the presence of cumene hydroperoxide. Results were reported as U/mg protein.

Lipid Peroxidation (MDA Levels)

Malondialdehyde levels, an index of lipid peroxidation, were quantified using the thiobarbituric acid reactive substances (TBARS) assay (Ghani et al. 2017). MDA concentration was expressed as nmol/mg protein.

Protein Determination

Total protein concentration in homogenates was determined using the Biuret method to normalize enzyme activities.

Histological Processing

Formalin-fixed duodenal tissues were dehydrated in ascending alcohol grades, cleared in xylene, and embedded in paraffin wax. Sections of 5 µm thickness were cut using a rotary microtome and stained with hematoxylin and eosin (H&E).

Slides were examined under a light microscope at ×40 and ×100 magnifications. Photomicrographs were captured for documentation and comparison of architectural changes including:

- mucosal villi integrity
- mucosal glands
- submucosa architecture
- ulceration or erosion
- muscularis mucosa and propria

Statistical Analysis

All data were expressed as mean ± standard error of mean (SEM). Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. Within-group comparison of initial and final body weight was carried out using paired t-test. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 25.0.

Results and Discussions

This study investigated the effects of alcohol, melatonin, and their combined administration on body weight, oxidative stress parameters, and duodenal histomorphology in Wistar rats. The findings demonstrate that chronic exposure to 50% alcohol induces significant oxidative and structural damage in the duodenum, while melatonin effectively protects against these deleterious effects.

At baseline, there were no significant differences in initial body weights across the experimental groups. After 28 days of treatment, paired comparisons showed a significant increase in final body weight within each group relative to its initial value ($p < 0.05$) as indicated in Table 1. When weight gain was compared across groups (Table 2), rats treated with 50% alcohol only exhibited a significant reduction in weight gain relative to the control group ($p < 0.05$). Conversely, animals treated with alcohol + melatonin demonstrated significantly greater weight gain compared with the alcohol-only group ($\#p < 0.05$), suggesting partial recovery of growth deficit by melatonin co-administration. Alcohol administration resulted in markedly reduced weight gain compared to the control group, consistent with previous studies showing that ethanol impairs nutrient absorption, increases metabolic burden, and suppresses appetite through gastrointestinal inflammation and mucosal damage (Butt et al., 2023). Chronic ethanol exposure is also known to interfere with intestinal motility and hormonal regulation, further contributing to poor weight outcomes (Keshavarzian and Fields, 2017). In the present study, co-administration of melatonin significantly improved weight gain relative to the alcohol-only group, indicating that melatonin supports metabolic stability and may mitigate alcohol-induced nutritional deficits.

Table 1. Body Weight Parameters - Within Group Comparisons (Initial vs Final)

Group	Initial Weight (g)	Final Weight (g)	p-value (Paired t-test)
Control	166.5 ± 0.9	187.0 ± 0.4	<0.001*
50% Alcohol	161.8 ± 4.2	171.5 ± 3.9	0.003
5mg/kg Melatonin + 50% Alcohol	173.3 ± 3.5	191.5 ± 2.1	<0.001*
5mg/kg Melatonin	165.5 ± 2.3	191.3 ± 1.2	<0.001*

Values are expressed as mean ± SEM. * $P < 0.05$ indicates statistical significance between initial and final weight within each group using paired t-test

Table 2. Weight Change Across Experimental Groups

Parameter/Group	Control	50% Alcohol	5mg/kg Melatonin + 50% Alcohol	5mg/kg Melatonin	p-value
Weight Change (g)	20.5 ± 0.5	4.8 ± 0.6 *	18.3 ± 1.6 #	25.8 ± 1.2 #	<0.001

Values are expressed as mean ± SEM. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the 50% alcohol only group based on Tukey's HSD post-hoc test following one-way ANOVA

The antioxidant assays provide further evidence of alcohol-induced oxidative stress. Rats treated with alcohol showed significant reductions in SOD, CAT, and GPx activities alongside elevated MDA levels, confirming excessive generation of reactive oxygen species (ROS) within the duodenum. These alterations are consistent with previous reports demonstrating that ethanol metabolism generates free radicals and disrupts the enzymatic antioxidant defense system (Contreras-Zentella et al. 2022). Lipid peroxidation, indicated by increased MDA levels, reflects membrane damage and plays a central role in ethanol-induced mucosal injury (Akinrinde and Ajibade 2024). Melatonin supplementation significantly reversed these biochemical alterations. Co-treatment restored SOD, CAT, and GPx activities and reduced MDA concentrations toward normal values. These findings align with established evidence that melatonin is a potent antioxidant capable of directly scavenging free radicals, upregulating endogenous antioxidant enzymes,

and stabilizing cellular membranes (García et al. 2014, Reiter et al. 2016, Hacışevki and Baba 2018). Melatonin also improves mitochondrial function and reduces the activation of inflammatory pathways, contributing to its protective effects in gastrointestinal tissues (Favero et al. 2017).

Table 3. Oxidative Stress Parameters in Gastric Tissue

Parameter/Group	Control	50% Alcohol	5mg/kg Melatonin + 50% Alcohol	5mg/kg Melatonin	p- value
SOD	1.68±0.06	0.58±0.08*	1.68±0.11#	1.73±0.11#	<0.001
CAT	1.29±0.01	0.28±0.02*	1.31±0.01#	1.33±0.01#	<0.001
GPx	1.49±0.04	0.79±0.05*	1.13±0.04*#	1.14±0.05#	<0.001
MDA	0.46±0.05	1.29±0.03*	0.36±0.02#	0.40±0.04#	<0.001

Values are expressed as mean ± SEM. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the 50% alcohol only group based on Tukey's HSD post-hoc test following one-way ANOVA

Histologically, alcohol-fed rats exhibited extensive mucosal damage characterized by villus erosion, glandular distortion, and funnel-shaped ulceration. This agrees with known patterns of ethanol-induced intestinal injury, where oxidative stress, inflammation, and epithelial apoptosis contribute to mucosal breakdown (Brzozowska et al. 2014). In contrast, animals treated with melatonin showed well-preserved villi, intact mucosal glands, and normal submucosal and muscular layers. The protective histological profile further supports melatonin's role in enhancing mucosal integrity, maintaining epithelial tight junctions, and promoting tissue repair (Karaaslan and Suzen 2015).

The melatonin-only group showed no biochemical or histological abnormalities, confirming its safety and lack of adverse effects on the gastrointestinal tract, a finding also supported by earlier toxicological studies (Kolli et al. 2013).

Overall, the combination of antioxidant restoration, improved weight gain, and preserved histoarchitecture strongly indicates that melatonin possesses significant therapeutic potential in mitigating alcohol-induced duodenal injury. This is particularly relevant because chronic alcohol consumption continues to be a major contributor to gastrointestinal morbidity worldwide.

Histology

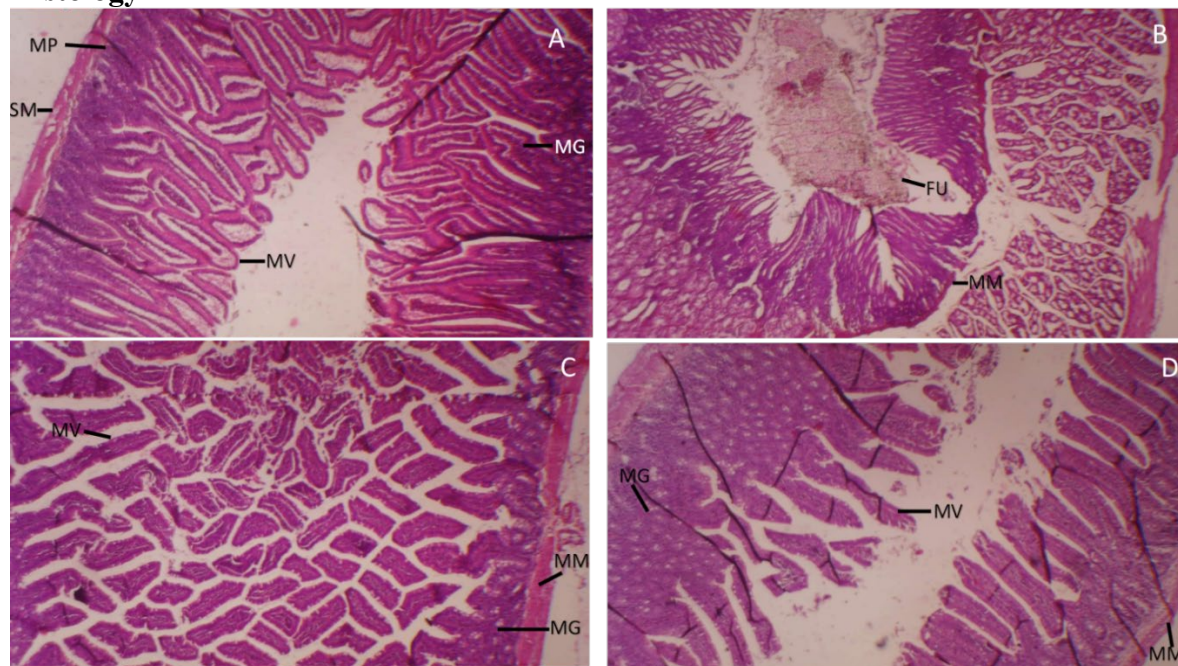


Figure 1. Plates A–D show sections of rat duodenum (H&E, 40X): (A) control with normal architecture comprising mucosal villi (MV), mucosal glands (MG), submucosa (SM), and muscularis propria (MP); (B) alcohol-treated showing a funnel-shaped ulcer (FU) and muscularis mucosa (MM); (C) alcohol + melatonin-treated with preserved normal architecture of MV, MG, and MM; and (D) melatonin-treated showing normal MV, MG, and MM

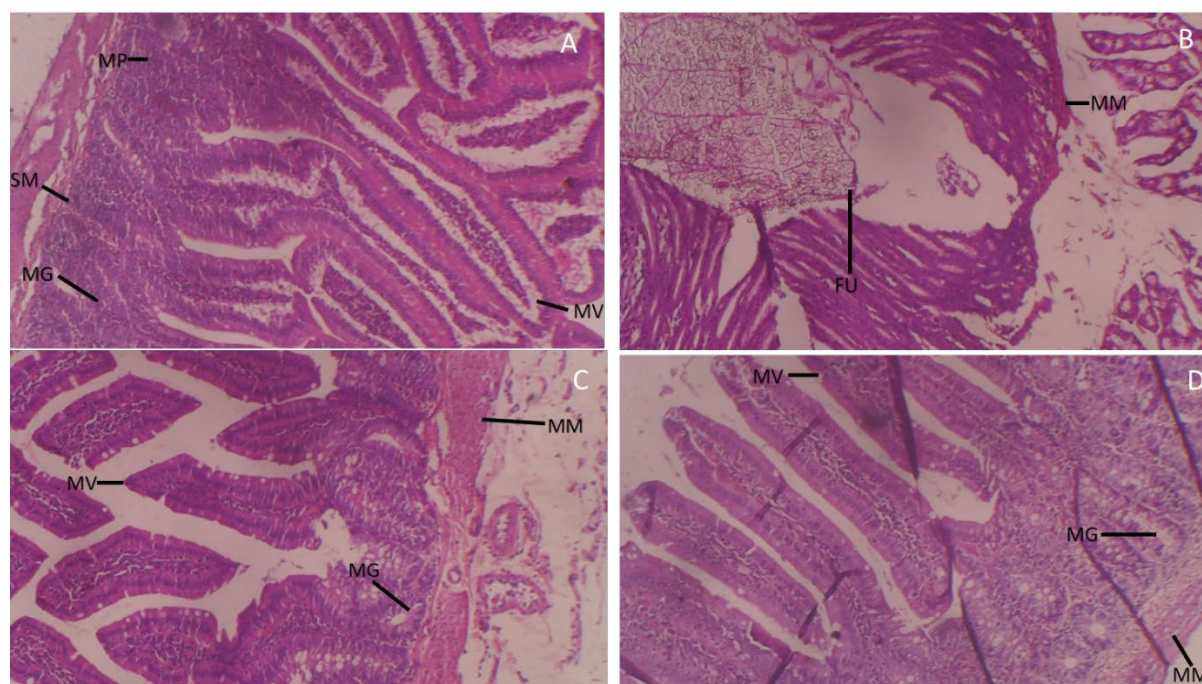


Figure 2. Plates A–D show sections of rat duodenum (H&E, 100X): (A) control with normal architecture comprising mucosal villi (MV), mucosal glands (MG), submucosa (SM), and muscularis propria (MP); (B) alcohol-treated showing a funnel-shaped ulcer (FU) and muscularis mucosa (MM); (C) alcohol + melatonin-treated with preserved normal architecture of MV, MG, and MM; and (D) melatonin-treated showing normal MV, MG, and MM

Conclusions

Chronic administration of 50% alcohol induced significant oxidative stress, impaired antioxidant defenses, reduced weight gain, and caused severe duodenal mucosal injury in Wistar rats. Melatonin co-administration effectively countered these effects by restoring antioxidant enzyme activities, reducing lipid peroxidation, preserving duodenal histological architecture, and improving weight outcomes. Melatonin alone produced no deleterious effects, underscoring its safety.

Melatonin therefore demonstrates strong gastroprotective potential against alcohol-induced duodenal toxicity and may represent a beneficial adjunct in preventing or managing alcohol-related intestinal injury.

Availability of data and materials

The data can be obtained from the corresponding author upon reasonable request by contacting them through their email ID.

Acknowledgements

None.

Funding

None.

Authors Contribution

Edobor Obayuwana: Methodology, Supervision and project administration Investigation, Resource. Joseph Raymond Enoghase: Data curation, writing review and editing and visualization. All authors have read and agreed to the published version of the manuscript.

Ethics declarations

Ethics approval and consent to participate

The research ethics committee's guidelines for animal handling and treatment at the University of Benin's College of Medical Sciences was fully implemented.

Consent for publication

Not applicable.

Competing interests

None.

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CARBOHYDRATE OXIDASE GENE EXPRESSION IN SUNFLOWER DURING *OROBANCHE CUMANA* INFECTION

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Abstract

Carbohydrate oxidases (SCO) represent a class of enzymes involved in carbohydrate metabolism and in the generation of hydrogen peroxide (H₂O₂), a key molecule in redox signaling and in the activation of plant defense responses. In this study, the transcriptional responses of the *SCO* gene were analyzed in three sunflower genotypes exhibiting contrasting reactions to *O. cumana* infestation, using a controlled artificial infestation system. The resulting expression profiles were correlated with the resistance or susceptibility phenotypes of the analyzed genotypes. The results revealed significant transcriptional differences among genotypes. Resistant genotypes showed an early and tightly regulated induction of *SCO* expression during the pre-attachment phase, followed by moderate and sustained activation associated with effective restriction of parasite establishment. In contrast, the susceptible genotype Performer exhibited a delayed and unbalanced response, insufficient to prevent successful infestation.

Keywords: carbohydrate oxidase (*SCO*), *Helianthus annuus* L., *Orobancha cumana* Wallr., post-attachment, pre-attachment, pathogen resistance, biotic stress

Introduction

Early defense responses in plant-pathogen and plant-parasite interactions are critically dependent on tightly regulated redox signaling (Lamb et al. 1997, Torres et al. 2006, Mittler et al. 2011). These responses are characterized by a rapid and transient accumulation of reactive oxygen species (ROS), particularly hydrogen peroxide (H₂O₂), which functions both as a direct antipathogen agent and as a central signaling molecule coordinating local and systemic immunity (Apel et al. 2004, O'Brien et al. 2012, Waszczak et al. 2018). While excessive ROS accumulation leads to oxidative damage and cellular dysfunction, controlled ROS production is essential for the activation of defense-related transcriptional networks, cell wall reinforcement, and the establishment of effective resistance (Bolwell et al. 2002, Suzuki et al. 2011). Moreover, maintaining ROS homeostasis is increasingly recognized as crucial not only for defense responses but also for regulating plant growth and development under both biotic and abiotic stresses (Lopez 2024, Xu 2024).

Carbohydrate oxidases (SCO) establish a direct mechanistic link between carbon metabolism and redox-mediated defense signaling (Bolouri-Moghaddam 2010). At the molecular level, H₂O₂ produced by these enzymes has been shown to activate pathogenesis-related (PR) genes, stimulate phytoalexin biosynthesis, and promote cell wall fortification via lignification and cross-linking of structural polymers (Wojtaszek 1997, Passardi et al. 2004, Hüeckelhoven 2007).

Evidence from model plant systems indicates that carbohydrate oxidase-encoding genes are rapidly induced following fungal or bacterial infection, as well as upon treatment with defense-associated phytohormones, including salicylic acid (SA), jasmonic acid (JA), and ethylene (Custers et al. 2004). In *Nicotiana tabacum*, carbohydrate oxidase activity has been directly implicated in the hypersensitive response (HR), where localized ROS accumulation contributes to programmed cell death and restriction of pathogen spread (Heath 2000, Custers et al. 2004). These findings support the concept that carbohydrate oxidases function as regulators of oxidative burst amplitude and timing rather than merely as metabolic enzymes (Mittler et al. 2004, Baxter et al. 2014).

SCO shares high sequence homology with a carbohydrate oxidase that exhibits antifungal activity and confers resistance to fungal pathogens when expressed in transgenic *Arabidopsis.SCO* (Hu et al. 2003). *SCO* shares high homology with the berberine bridge enzyme (BBE) (Lu 2003). These oxidases can transfer a hydride to the FAD cofactor, facilitating complex substrate transformations such as cyclizations, C–C bond formation, and nucleophilic additions (Tjallinks 2024). For example, BBE-like enzymes in *Arabidopsis* contribute to plant defense by oxidizing cell wall-derived oligosaccharides, such as oligogalacturonides and cellodextrins, thereby reducing their activity as damage-associated molecular patterns, limiting *Botrytis cinerea* growth, and helping maintain immune homeostasis (Locci 2019). Similar carbohydrate oxidases have been described as antifungal proteins in sunflower (*Helianthus annuus*) and lettuce (*Lactuca sativa*) (Custers 2004). However, most studies to date have focused on plant-fungi interactions, whereas the role of these enzymes in plant-plant parasitic interactions, as well as in the timing and regulation of plant defense responses and associated gene expression, remains poorly understood.

This knowledge gap is particularly relevant in the context of the *Helianthus annuus* L. and *Orobancha cumana* Wallr. interaction. *O. cumana* is a holoparasitic plant that establishes a haustorial connection with sunflower roots, enabling the extraction of water, carbohydrates, and other metabolites (Fernández-Melero et al. 2024, Auriac 2024). Successful parasitism requires extensive remodeling of host cell walls and manipulation of host carbon fluxes, processes that are strongly influenced by host redox status and ROS signaling (Hegenauer et al. 2017). Transcriptomic analyses of resistant sunflower genotypes have consistently reported early activation of redox-associated genes, enhanced ROS signaling, and reinforcement of host cell walls, whereas susceptible genotypes exhibit delayed, excessive, or poorly coordinated oxidative responses (Letousey et al. 2007, Louarn et al. 2016).

Despite these findings, the transcriptional dynamics of *SCO* during the different stages of *O. cumana* infection, and its association with host resistance or susceptibility, remain insufficiently studied.

Therefore, investigating *SCO* expression dynamics in sunflower genotypes with contrasting resistance phenotypes provides an opportunity to clarify the role of redox-regulated carbohydrate oxidation in host-parasite interactions. Such insights may not only advance the mechanistic understanding of sunflower resistance to *O. cumana* but also support the identification of redox-related molecular markers for breeding durable resistance.

The present study investigates the temporal and genotype-dependent regulation of a sunflower carbohydrate oxidase gene (*SCO*) during two critical phases of host-parasite interaction: (i) the pre-attachment stage, corresponding to early perception and signaling events, and (ii) the post-attachment stage, associated with haustorial penetration and sustained defense responses.

Materials and Methods

In this study, three sunflower genotypes (Favorit, PR64LE20, and Performer) provided by the National Agricultural Research and Development Institute (NARDI) Fundulea, Romania, were

evaluated. According to NARDI data and the specialized literature, the Favorit genotype carries the Or6 gene, which confers specific resistance against race F of the parasitic plant *Orobancha cumana*. The PR64LE20 genotype is characterized by a high level of differential resistance, showing resistance to races A-G of the pathogen. The Performer genotype does not contain *Or* genes associated with resistance and exhibits high susceptibility to *O. cumana* infestation. For the establishment of the artificial infestation background, broomrape seeds collected in 2014 from Singera, Republic of Moldova, characterized by a high aggressiveness toward sunflower genotypes, were used (Duca 2017, Tabara 2020). Only broomrape seeds with a germination rate $\geq 80\%$, verified in preliminary tests for viability - Figure 1 A-D and germination capacity - Figure 1 E-G, were used in the experiment.

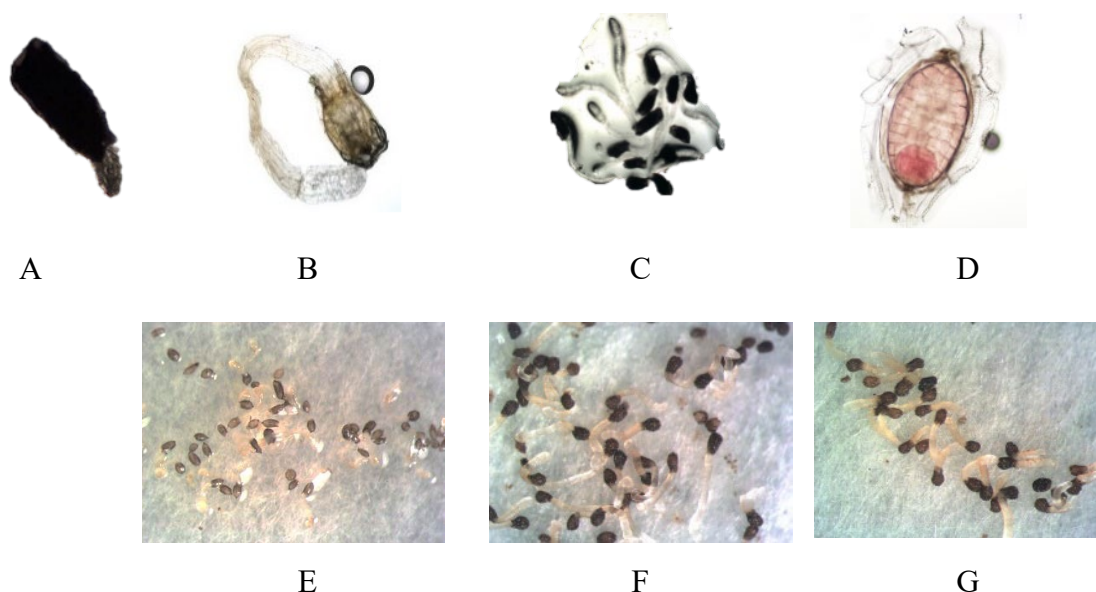


Figure 1. Germination of *Orobancha cumana* seeds tested in water (A), sunflower root exudate (B), GR24 (C), tetrazolium chloride (D), and at germination levels of 25% (E), 50% (F), and 75% (G)

To characterize the transcriptional profile of the *SCO* gene within defense mechanisms, two experimental systems were used, designed to both immediate responses, illustrated in Figure 2A and medium- to long-term molecular changes, presented Figure 2B.

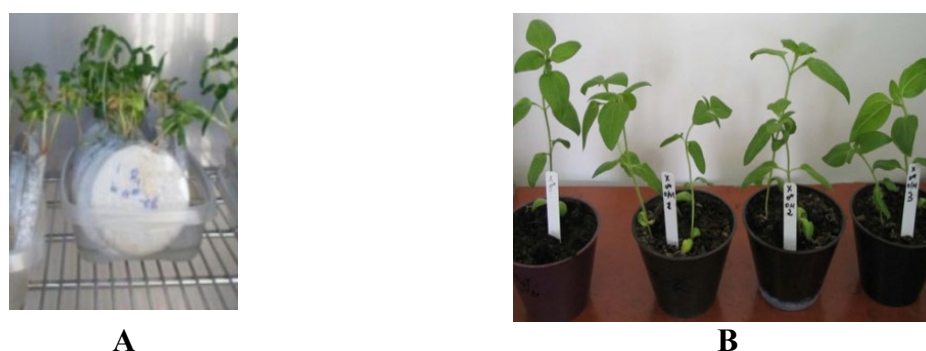


Figure 2. Cultivation of sunflower against an infection background in Petri dishes (A) and in pots (B).

The first experimental system involved cultivating sunflower plants in Petri dishes on a perlite substrate (rhizotron model) in a germination chamber at 20-25 °C under a 16 h light / 8 h dark photoperiod Figure 2.A, allowing for the evaluation of early root responses associated with pre-

attachment mechanisms. Pre-germinated (on exudate) broomrape seeds were applied to sunflower root grown *a priori* until two-leaf stage. To monitor the dynamics of gene expression, samples were collected after 2, 6, 12 and 24 h (post-infestation: *hpi*) of host-parasite co-cultivation.

The second experimental system was designed to analyze the host-parasite response under conditions closer to the natural environment and involved the cultivation of genotypes in pots (Figure 2.B). Substrate infestation was performed at a rate of 37 mg of *O. cumana* seeds per 200 g of soil mixture. Sunflower roots samples were collected at the critical developmental phases of the pathosystem, namely the formation of the first attachments (18–21 days), tubercle development (35 days), underground shoot formation (53 days), and emergence of aerial shoots (67 days).

Uninfested substrate-grown genotypes were used as controls. Biological material was collected from three biological replicates, frozen in liquid nitrogen, and subsequently stored at -80°C .

RNA isolation and cDNA synthesis

Total RNA was extracted from root tissues of each of the three plants using the TRI-zol method (Applied Biosystems). RNA quality and quantity were assessed via spectrophotometry (260/280 nm) and 1% agarose gel electrophoresis. RNA was treated with DNase (Thermo Scientific) prior to cDNA synthesis, which was carried out using Revertaid.

RT with Oligo-dT18 and random hexamer primers

Analysis of relative *SCO* gene expression. *SCO* gene expression levels were determined by real-time PCR using a DT-96 amplifier (DNA Technology, Russia). Specific primers were designed based on EST sequences available in the NCBI database using the Primer3Web v.3.0.0 and OligoAnalyzer 3.1 bioinformatics tools. Actin (AF282624.1) was used as the reference gene (Table 1).

Fluorescence detection was performed with Maxima SYBR Green/ROX PCR Master Mix using 0.4 μM primers and 2 μL of cDNA per reaction. The amplification program included an initial denaturation at 95°C for 10 min, followed by 5 cycles of 95°C for 15 s and 64°C for 20 s, then 40 cycles of 95°C for 15 s and 60°C for 40 s. The absence of nonspecific amplicons and primer dimers was verified by 1.4% agarose gel electrophoresis and melting curve analysis.

Table 1. Nucleotide sequences of the primers

Nr.	Gene	Access ID	F Primer	R Primer	Amp.
1.	carbohydrate oxidase	AF364866.1	gggtggacttcgtggatcag	ctcgggttactcgtcacgta	112 pb
2.	actin (reference gene)	AF282624.1	gctaacagggaaaagatgactc	actggcataaagagaaagcagc	96 pb

qPCR reactions were performed in three technical replicates for each of the three biological replicates. The obtained results were statistically processed by calculating the following parameters: arithmetic mean ($\bar{x}$), variance (s^2), standard deviation (s), coefficient of variation (CV), and standard error of the mean (SEM). All data are presented as mean $\pm$ SEM. Differences were evaluated using the t-test. All these indices were calculated to assess data variability, the precision of estimates, and significant differences between experimental variants.

Normalized expression levels were calculated using the $2^{-\Delta\text{Ct}}$ method, where $\Delta\text{Ct} = (\text{Ct}_{\text{target gene}} - \text{Ct}_{\text{reference gene}})$. To compare gene expression between plants grown in the presence of broomrape seeds and control plants, fold changes were determined using the $2^{-\Delta\Delta\text{Ct}}$ method. When $2^{-\Delta\Delta\text{Ct}} < 1$, downregulation of gene expression was expressed as the negative reciprocal value ($-1/2^{-\Delta\Delta\text{Ct}}$), representing the fold reduction in gene expression (Schmittgen and Livak, 2008). Fold changes with values ≤ -1.5 or ≥ 1.5 were considered biologically relevant for evaluating the defensive response of the genotypes to *O. cumana* infestation. Statistical significance of differences in gene expression was assessed using Student's t-test, and differences were considered statistically significant at $p \leq 0.05$.

Results and discussions

SCO expression in non-infested plants.

In the absence of *Orobanche cumana*, *SCO* transcript levels during the pre-attachment phase followed a comparable temporal pattern across all three sunflower genotypes, as illustrated in Figure 3A, characterized by a transient decrease at 6 and 12 hpi and recovery at 24 hpi. This behavior is consistent with the dynamic regulation of ROS-related genes under basal growth conditions, reflecting their involvement in developmental redox signaling rather than in stress-induced responses (Apel 2004, Mittler et al. 2004, Waszczak et al. 2018). Despite this shared trend, clear genotype-dependent differences in expression magnitude were evident. The resistant genotypes Favorit and PR64LE20 maintained relatively stable and moderate *SCO* transcript levels, whereas the susceptible genotype Performer displayed significantly higher expression at early time points.

In the post-attachment experimental system (18–67 days), non-infested plants again displayed genotype-specific *SCO* expression dynamics as displayed in Figure 3B.

Oscillatory expression patterns observed in Favorit and PR64LE20 suggest a tightly regulated redox network, in agreement with reports describing coordinated ROS production and scavenging during plant development (Bolouri-Moghaddam et al. 2010, Waszczak et al. 2018). In contrast, the early high *SCO* expression followed by attenuation in Performer supports the notion of inefficient redox control, which may predispose this genotype to ineffective stress responses.

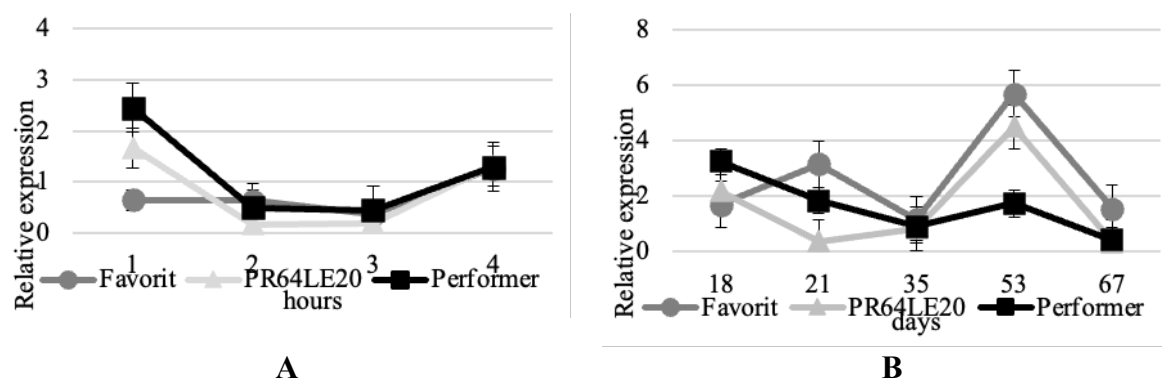


Figure 3. Relative expression of the *SCO* gene in sunflower hybrids grown under standard conditions (without *Orobanche* seeds) in Petri dishes (A) and in pots (B)

This common regulatory trend suggests that *SCO* expression is dynamically regulated even in the absence of parasite-derived signals.

SCO expression in infested plants during pre-attachment (*first experimental system*).

During the pre-attachment phase of *O. cumana* infection, *SCO* expression exhibited clear genotype-dependent modulation, is shown in Figure 4 and Figure 5.

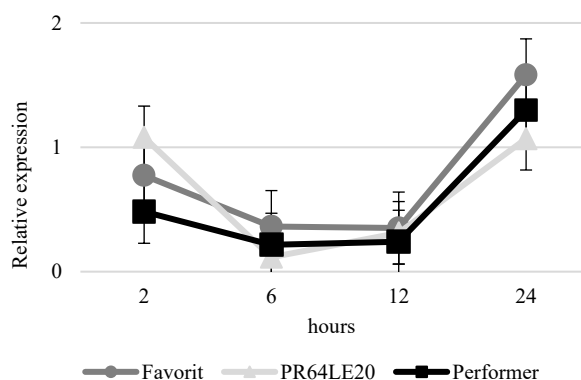


Figure 4. Relative expression of the *SCO* gene in sunflower hybrids under artificial infestation in first experimental system

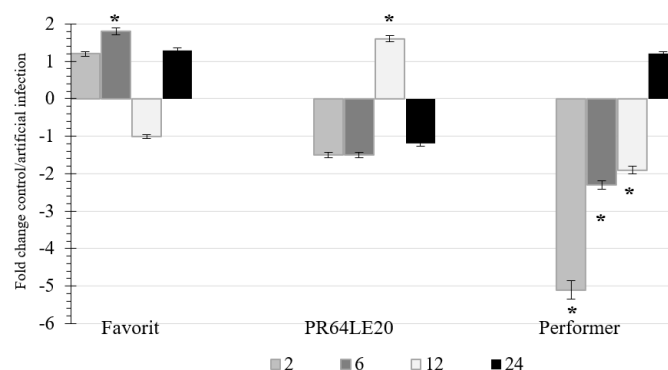


Figure 5. *SCO* gene expression levels (fold change) in different sunflower genotypes under artificial *O. cumana* infestation. An asterisk (*) indicates a statistically significant difference at $p \leq 0.05$ (*t*-test)

The reduced levels of *SCO* transcripts at 2 and 6 hpi in the majority of genotypes compared with control samples indicate a transient modulation of ROS signaling following parasite recognition, resembling the early oxidative burst adjustments reported in other host-pathogen systems (Lamb 1997, Wojtaszek 1997). The subsequent induction at 24 hpi was observed in all genotypes.

Contrary to the findings of Letousey et al. (2007), who reported similar expression patterns in both resistant and susceptible sunflower genotypes during the early induction phase, our study revealed distinct transcriptional responses between resistant and susceptible genotypes. Thus, in resistant hybrids, *SCO* expression changes remained moderate and statistically insignificant relative to controls, indicating a restrained response maintained within a functional reaction norm.

In contrast, the susceptible genotype Performer showed significant early repression followed by late overexpression of *SCO*, a pattern indicative of delayed and poorly coordinated defense activation.

Thus, the resistant hybrids Favorit and PR64LE20 exhibit a distinct response to artificial infection, with no statistically significant changes in fold change compared with the control, while the susceptible hybrid Performer displays a time-dependent response, characterized especially by the subexpression of analyzed gene.

Overall, the figure highlights clear genotype-dependent differences in *SCO* gene expression under *O. cumana* infestation, with Favorit showing a transient response, PR64LE20 and Performer exhibiting a moderate, respectively, strong downregulation.

***SCO* expression in infested plants during post-attachment (second experimental system).** During post-attachment stages, *SCO* transcriptional dynamics further emphasized genotype-dependent defense strategies, is presented in Figure 6 and Figure 7.

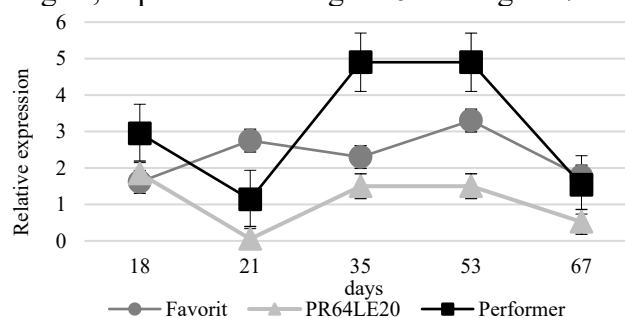


Figure 6. Relative expression of the *SCO* gene in sunflower hybrids under artificial infestation in second experimental system

Resistant genotypes exhibited coordinated phases of *SCO* induction and repression corresponding to key developmental stages of the parasite. The observed repression of *SCO* in PR64LE20 during haustorial attachment and underground shoot formation suggests an active regulation (Bolwell et al. 2002, Bindschedler et al. 2006, O'Brien et al. 2012).

In contrast, the susceptible genotype showed delayed but strong *SCO* overexpression at later stages, likely reflecting stress-induced ROS accumulation rather than an effective defense response. Late ROS bursts have been shown to coincide with irreversible tissue damage and unsuccessful defense outcomes (Heath 2000, Hüchelhoven 2003).

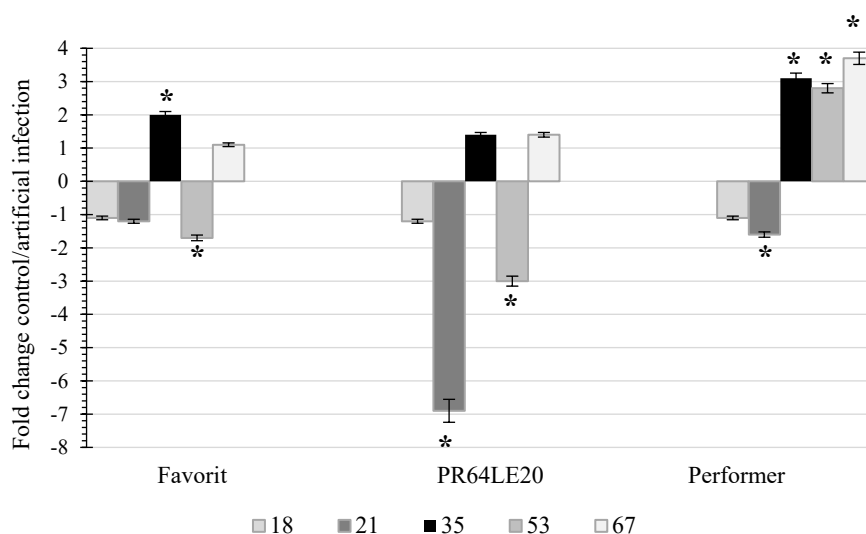


Figure 7. Fold-change expression of the *SCO* gene in different sunflower genotypes under artificial *Orobancha cumana* infestation. An asterisk (*) indicates a statistically significant difference at $p \leq 0.05$ (t-test)

These findings align with previous studies on sunflower – *Orobancha* interactions, which demonstrated that resistance is associated with early, localized responses, whereas susceptibility correlates with delayed systemic reactions (Letousey et al. 2007, Louarn et al. 2016).

Overall, the results obtained in the second experimental system corroborate those observed in the first system, confirming that *SCO* transcriptional regulation is strongly influenced by genotype.

The resistant sunflower genotypes Favorit and PR64LE20 employ distinct redox-based strategies to limit *Orobancha cumana* infection. Favorit displays moderate, well-timed *SCO*

activation at critical developmental stages, supporting ROS-mediated signaling and structural defenses, whereas PR64LE20 maintains predominantly repressed *SCO* expression, restricting excessive apoplastic ROS while preserving effective defense (Bolwell et al., 2002, Apel 2004, Passardi et al. 2004, Mittler et al. 2011). In contrast, the susceptible genotype Performer exhibits constitutively high basal *SCO*, followed by early repression and delayed, excessive late induction upon infestation, reflecting poorly coordinated defense signaling and stress-induced ROS accumulation after parasite establishment (Heath 2000, Hükelhoven 2003, Waszczak et al. 2018).

These observations indicate that resistance depends on precise temporal regulation of ROS-related genes, rather than on maximal ROS production, highlighting the importance of coordinated redox homeostasis in sunflower defense (Torres et al. 2006, Letousey et al. 2007, Suzuki et al. 2011). This concept aligns with current models of plant immunity, where finely tuned ROS signaling networks, rather than excessive oxidative bursts, underpin durable resistance (Mittler et al. 2011, Suzuki et al. 2011, Waszczak et al. 2018). The deregulated and energetically inefficient *SCO* expression observed in the susceptible genotype likely contributes to its failure to restrict *O. cumana* development. Similar defense mechanisms have been documented in sunflower lines overexpressing ROS-generating enzymes, which show enhanced resistance responses (Hu et al. 2003).

Conclusions

The results indicate that the transcriptional dynamics of *SCO*, rather than its absolute expression levels, may be associated with sunflower responses to *Orobanche cumana*. The balance between ROS production and antioxidant control appears to contribute to resistance mechanisms. Overall, *SCO* may represent a candidate gene for further investigation as a potential molecular marker in resistance breeding programs

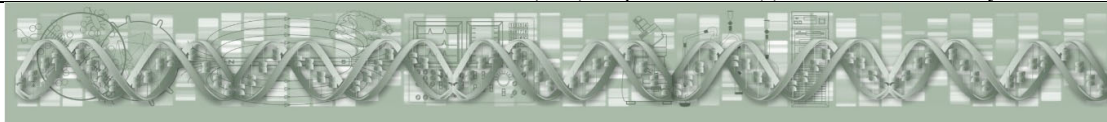
Acknowledgments: The research was carried out within the Subprogram 011101 - *Genetic and biotechnological approaches to agroecosystem management under climate change*, funded by the Ministry of Education and Research of the Republic of Moldova.

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MICROBIAL VALORIZATION OF NICOTINE AND TOBACCO WASTE FOR BIOACTIVE COMPOUNDS RECOVERY: A SYSTEMATIC REVIEW

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Abstract

The tobacco industry produces thousands of tons of toxic waste annually, containing nicotine at concentrations ranging from 3% to 6% (w/w). Traditional waste management approaches entail high costs and negative impacts on the environment and human health. Microbial biotechnology may be used for the simultaneous detoxification of nicotine and recovery of bioactive compounds from tobacco waste (TW). The systematic review is based on the guidelines established by PRISMA 2020 and seeks to find data on bacteria-based approaches to TW valorization, structured around the PICO framework. The literature search was conducted between April and June 2026 across Scopus, Web of Science (WoS), PubMed, and Google Scholar, supplemented by reports from World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), and Grand View Research. Following duplicate removal and screening of 201 studies, 13 research articles and 7 organizational reports met the inclusion criteria. Of the 13 studies, 7 focused on NDM strains, 4 on nicotine-tolerant non-NDMs, and 2 on co-culture systems. Functional pyridines were the most frequently recovered compounds, with pharmaceutical applications being the predominant target sector. The findings confirm that microbial valorization of TW aligns well with circular economy principles. Nevertheless, some limitations must be acknowledged: the screening process was conducted by a single author, and the considerable heterogeneity of experimental conditions across the included studies precluded a quantitative meta-analysis, making a narrative synthesis the only viable approach. Future research should prioritize scale-up studies and safety validation of the produced compounds.

Keywords: nicotine, tobacco waste, microbial valorization, bioactive compounds, circular economy

Introduction

The tobacco industry is one of the largest and most controversial markets in the world. Its global market venue was estimated at 926 billion USD in 2025 (1), while according to WHO, annual cost of treatment and productivity loss associated with smoking is higher than 1 trillion USD (2). Tobacco kills around 7 million people around the world and 1.1 million people each year in the European Union (EU). People who smoke have 2 - 4 times higher risks of having heart disease and suffering a stroke than nonsmokers (3, 4). However, tobacco consumption in the past ten years has increased by 25% - 30%. The increase in consumption is particularly high among young population. The reason behind such trend is aggressive marketing strategy



applied by the industry and introduction of new heated tobacco products (HTPs). In general, HTPs are presented as much healthier alternatives to cigarette smoking. These factors led to an increase in tobacco mortality of 15.4% among men and 4.7% among women in EU (3).

For most people, the harm caused to society by the tobacco industry is primarily associated with health issues. However, the environmental impacts it generates are rarely taken into consideration. The process of farming tobacco is very intensive since it requires high amounts of pesticides and fertilizers that are extremely toxic chemicals that pollute the groundwater (5, 6). The largest tobacco producing nations include China, India, Brazil, the United States, countries across Southeast Asia, and those in sub-Saharan Africa. Worldwide, the production of tobacco plants is estimated to cover about 4.3 million hectares of land, while 200.000 hectares of forest are felled yearly in order to grow and cure tobacco. This results in deforestation at 2% - 4%, which is equivalent to the loss of about 600 million trees per annum (1, 5, 6). Additionally, 22 billion tons of water are consumed for irrigating crops annually, while the production chain generates around 2 million tons of solid waste each year, including 300.000 tones of nicotine-contaminated residues and 200.000 tones of chemical by-products, making TW particularly difficult to manage and dispose of safely (5–7).

The annual cost involved in the treatment of TW reach 2.6 billion USD in China, 766 million USD in India, 234 million USD in Germany and over 200 million USD in Brazil (6). In response, WHO has called on countries to prioritize tobacco control as part of their commitments to the 2030 Agenda for Sustainable Development Goals (5).

Traditional approaches such as disposal through landfills, burning, pyrolysis, gasification, and chemical solvent neutralization not only come at a cost but are also harmful to the environment (7). In addition to its impact on health, the large amount of waste generated annually by the tobacco industry also represents an untapped resource that, if used efficiently, could contribute significantly to the adoption of a circular economy principles in this sector.

Recently, an increasing number of studies have been published focusing on research into various environmentally friendly methods for recycling TW. In particular, the use of NDMs represents one of the most promising approaches. NDMs are capable of using nicotine as a sole source of carbon and energy, playing a role in the bioremediation of TW by neutralizing nicotine toxicity. Nicotine degradation involves several metabolic pathways (pyridine, pyrrolidine, and VPP) which yield functional pyridines with potential for use in the production of bioactive compounds applicable in different domains. In addition to NDMs, other bacterial strains capable of tolerating nicotine without degrading it have also shown considerable biotechnological potential for the valorization of TW, contributing to the recovery of commercially relevant compounds.

However, the current scientific literature on this topic is fragmented, and the integration of these techniques has not yet been well systematized. In this context, the main goal of this systematic review is to explore the most significant existing scientific findings regarding the application of bacterial biotechnology in the biological valorization of waste generated by the tobacco industry, with a special focus on the recovery of bioactive compounds that can be used in the pharmaceutical, agricultural, environmental chemistry, and food industries.

Materials and Methods

Search strategy

The systematic literature review was performed according to the PRISMA 2020 guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses). The PRISMA 2020 checklist, provided in Appendix 1, was used to guide the reporting and organization of this systematic review. These guidelines were used to define the search strategy, select databases, identify keywords for the search, and establish inclusion and exclusion criteria (8, 9).

The literature research was conducted from April to June 2026 and involved two research areas. The literature related to the scope of the tobacco industry worldwide and its adverse impact on the environment and human health, served as background information for the introduction section, was identified through the WHO, the FAO, and Grand View Research. The relevant literature on the valorization of nicotine and TW, and recovery of bioactive compounds using bacterial methods was conducted in four international databases: Scopus, WoS, PubMed, and Google Scholar.

The following Boolean operators were used to create various combinations of the chosen keywords within search strings: 'tobacco waste AND circular economy', 'tobacco waste AND nicotine-degrading microorganisms', 'nicotine AND microorganisms', 'tobacco waste OR tobacco residues', 'nicotine detoxification OR nicotine decontamination', 'circular economy AND bioactive compounds AND nicotine-degrading microorganisms', 'tobacco waste AND nicotine degradation'. Search was applied to the title, abstract, and keyword sections of indexed articles whenever possible. In addition, the reference list of the selected studies was screened to identify additional relevant publications. All the obtained documents were collected in a single Excel worksheet (Microsoft Corporation, SUA), and duplicates were manually removed.

Inclusion and exclusion criteria

The systematic review followed the PICO methodology as indicated into the PRISMA 2020 guidelines: *Population* – nicotine and TW; *Intervention* – biotechnological bacterial techniques, namely NDMs, novel NDMs, nicotine-tolerant non-NDMs, and co-cultures comprising both NDMs and non-NDMs; *Comparison* – no specific comparison among the investigated approaches was performed, as the included studies were evaluated individually through a narrative synthesis approach; *Outcome* – recovery of bioactive compounds with different industrial applications.

Studies were included if they satisfied the following criteria: i. reports containing data on the global scale of the tobacco industry and the associated human health and environmental issues, ii. original research articles published after 2014 in journals with an impact factor (IF) ≥ 1.5 , iii. studies focused on the identification of NDMs or other bacterial approaches for the recovery of valuable bioactive compounds from nicotine and TW, and iv. articles written in English, with the full text available. Studies were excluded if they met any of the following criteria: lack of empirical experimental data, articles focused exclusively on NDMs classification or the description of metabolic pathways without biotechnological relevance, genomic analysis studies, standard decontamination methods (pyrolysis, incineration, and physical-chemical neutralization), decontamination methods that did not focus on the recovery of bioactive compounds, and studies involving fungi in the decontamination of nicotine or TW.

Study selection and data extraction

Each paper was manually reviewed by a single author based on predefined criteria described in the section above. The studies were evaluated with regard to microbial bacteria-based approaches for the valorization of nicotine and TW, with a focus on the biotechnological recovery of valuable bioactive compounds and their potential applications in pharmacy, agriculture, and green chemistry, in line with the principles of the circular economy. The results were organized thematically according to the bacterial approach employed, distinguishing among NDM applications, novel NDM applications, nicotine-tolerant non-NDMs, and co-culture systems. No formal assessment of risk of bias was performed.

AI-assisted tools

Artificial intelligence-assisted tools were used during the preparation and organization of this systematic review. NotebookLM (Google, 2025) was used to synthesize and organize the key ideas from the included studies. DeepL (DeepL GmbH, Germany) was used for the initial translation of the selected content, while Claude Sonnet 4.6 (Anthropic, USA) was used to

support subsequent linguistic refinement and manuscript improvement. All content was critically reviewed and validated by the author.

Results

The total number of reports obtained from international organizations and market research companies was 22, while another 225 articles were found in international academic databases. This distribution is as follows: WHO (n = 13), FAO (n = 8), Grand View Research (n = 1), Scopus (n = 41), WoS (n = 72), PubMed (n = 57), and Google Scholar (n = 55). After removing duplicates, a total of 201 documents were screened for relevance, of which 17 were organizational reports and 184 were academic articles from international databases. Following this stage, a total of 6 organizational reports and 81 research articles that did not satisfy the inclusion criteria were excluded. During the secondary screening phase, an additional 4 organizational reports and 90 articles from databases were excluded.

In the final stage, 7 organizational reports and 13 research articles satisfied all inclusion criteria and were included in the qualitative review. The organizational reports were retrieved from the WHO (n = 6) and Grand View Research (n = 1), while the research articles were identified through Scopus (n = 2), PubMed (n = 7), and Web of Science (n = 4). PubMed provided the most specific studies on microbial degradation of nicotine, NDMs characterization, and metabolic pathways. The articles from Scopus included molecular investigations of the subject, as well as of the genome and proteome of NDMs. The largest variety of articles was generated by WoS, these covered various aspects of TW valorization, including the application of the circular bioeconomy using NDMs or different bacterial approach. As a final result, Google Scholar was used as a supplementary source for screening, however no additional eligible studies were identified for inclusion. The general study selection process is illustrated in the PRISMA 2020 flow diagram (Figure 1), assuring transparency and reproducibility in accordance with systematic review standards.

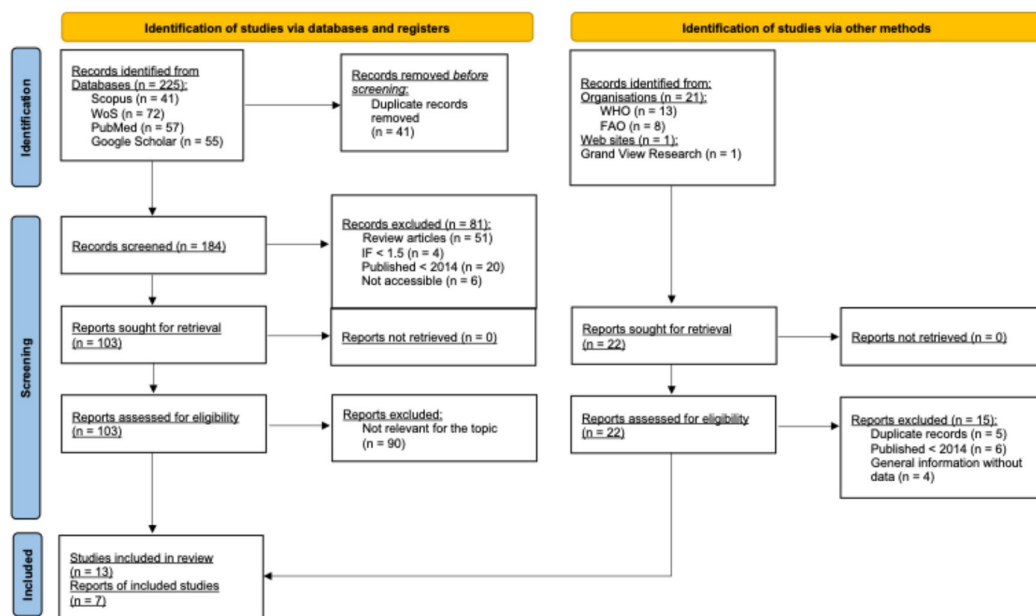


Figure 1. PRISMA 2020 flow diagram for systematic review

Out of the 13 studies included in this systematic review, 7 have focused on NDMs, 4 investigated nicotine-tolerant non-NDMs, and the other 2 examined co-cultures systems. In terms of compound classes, functional pyridines appear to be the most common one among the reviewed papers ($n = 7$). Furthermore, the pharmaceutical industry has been mentioned as the major industrial sector in 9 out of 13 cases. The selected articles were published between 2014 and 2026. As observed from the increasing trend of published articles starting from 2019, the interest of scientists towards biotechnology applications of TW appears to be increasing. A comprehensive overview of the included studies is provided in Appendix 2.

NDM applications

Nicotine released into the environment as a result of tobacco industry activities has been classified as a hazardous chemical in the Toxic Release Inventory (TRI) by the U.S. Environmental Protection Agency, with concentrations in solid and liquid waste ranging from 3% to 6% (w/w). Due to its high-water solubility, nicotine leached from TW can easily migrate into groundwater. Numerous studies report that it has been detected with increasing frequency in leachate and runoff from landfills. Therefore, the development of effective methods for removing nicotine from the large quantities of waste generated by the tobacco industry has become imperative. In this context, a series of bioremediation processes have been developed to reduce the nicotine content in TW using more environmentally friendly approaches than conventional decontamination methods, while also recovering end products with added economic value (10).

Based on the biotechnological potential of TW and the ways in which it can be exploited, genetic engineering is rapidly advancing, with the aim of optimizing the metabolic pathways for nicotine degradation and recovering metabolites of interest produced by NDMs. In recent years, bioremediation based on NDMs, isolated enzymes, or whole cells used as biocatalysts has become a promising solution, and several strains have attracted attention due to their remarkable efficiency. These include primarily strains belonging to the genera *Pseudomonas* and *Agrobacterium*, known for their ability to decontaminate nicotine through pyrrolidine and VPP pathways and produce bioactive compounds with applications in various industrial sectors. One of the major applications is the production of functional pyridines, used as precursors for the synthesis of various pharmaceuticals, including epibatidine analogues (an analgesic molecule), vitamin B6 (pyridoxine), analgesics (propiram, epibatidine), analeptics (nikethamide, camphotamide), and anti-inflammatory drugs (clonixin, nicoboxil). For example, compounds derived from nicotine metabolism that could be used directly in other applications include: 3-succinoyl-pyridine (SP) can be used as a precursor for the biosynthesis of anti-hypertensive agents (ω -heteroaroyl-(propynyl)-L-proline) (10), 6-hydroxy-3-succinoyl-pyridine (HSP) serves as a precursor for pioglitazone (for diabetes), eszopiclone (for insomnia), and crizotinib (for pulmonary cancer) (11), while 6-hydroxynicotine (6HN) can be used as a precursor for drugs designed to reduce symptoms of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (12).

***Pseudomonas putida* strains**

The following studies propose the development of efficient biotechnological strategies for converting nicotine from TW into high-value chemical compounds such as 3-succinoyl-pyridine (SP) and 6-hydroxy-3-succinoyl-pyridine (HSP), using engineered strains of *Pseudomonas putida*.

The first study employs an *in vitro* approach based on enzymes previously expressed in *E. coli* and purified by chromatography. This enzymatic "assembly line" consists of NicAm, Pnao, and Sapd – from *P. putida* – for the sequential conversion of nicotine into SP. In this biocatalytic enzymatic cascade, NicAm (nicotine oxidoreductase) initiates the pathway by oxidizing nicotine to pseudooxynicotine (PN), which is then transformed into 3-succinoyl-semialdehyde-pyridine (SAP) by Pnao (pseudooxynicotine amine oxidase). The final step is catalyzed by Sapd

(3-succinoyl-semialdehyde-pyridine dehydrogenase), which oxidizes SAP to produce the target product, SP. This sequence is assisted by an AKR module (aldehyde-ketone reductase) that ensures efficient and continuous regeneration of the NADP^+ cofactor required for the Sapd reaction, thereby maintaining a high catalytic flux. To optimize this process, the researchers employed spatial organization techniques through protein self-assembly using the SpyCatcher/SpyTag system and their site-specific immobilization on agarose microspheres activated by biotinylation mediated by the AviTag-BirA system. The biotinylation method is essential because the BirA enzyme attaches a biotin as an anchor tag to the AviTag segment at a fixed and specific point (a lysine residue), allowing the enzymes to be anchored onto the streptavidin support and co-immobilized into agarose microspheres without damaging their structure or blocking their active sites. This proximity-based molecular architecture reduces the diffusion distance of metabolites between neighboring enzymes and increases the nicotine conversion rate to 63%, a value significantly higher than the 42.7% achieved by free enzymes. Ultimately, the system reached a yield of approximately 70% (obtaining 0.35 mM SP from 0.5 mM nicotine), representing a 1.6-fold increase in productivity compared to the non-immobilized system. Beyond efficiency, this biocatalyst offers exceptional thermal stability, retaining over 50% of its activity even after exposure to a critical temperature of 70°C, while free enzymes lose 90% of their capacity under the same conditions. The robustness of the system allows its reuse over eight consecutive cycles with activity retention above 60%, with laboratory tests confirming that over 87% of the enzymatic proteins remain stably bound to the agarose microspheres after repeated use (13).

The following two studies use whole cells as biocatalysts, where they were introduced into water containing nicotine or tobacco waste extracts (TWE) to produce essential chemical compounds, representing a sustainable and faster alternative to conventional chemical methods. In parallel with complex enzymatic systems, such as the study described above, this study focused on modifying the *P. putida* S16 strain to generate the P-HSP variant. By knocking out the *hspB* gene via homologous recombination, the degradation pathway of HSP into 2,5-dihydroxypyridine (2,5-DHP) was blocked. This approach allowed for the production of very large quantities of HSP, reaching 16.3 g/L from pure nicotine and 6.8 g/L using TWE, with performance 3.7 times higher than that of the wild-type strain. The efficiency was remarkable, with the P-HSP strain managing to convert 99.4% of the nicotine in just 5 hours, while the unmodified strain reached only 38.9% in the same time interval. This method offers several remarkable advantages: a rapid and simple reaction system consisting of nicotine-containing water as both the cell support and substrate, which facilitates faster purification of the target compound, as well as the ability to reuse the cells across multiple production cycles. All in all, these features make whole-cell biocatalysis a cost-effective, scalable, and environmentally friendly strategy for the biotechnological valorization of TW, with strong potential for industrial application in the pharmaceutical and green chemistry industries (14).

The third study details a sustainable approach for converting TW into SP using the genetically engineered strain *P. putida* S16 Δ *spm*. This strain was obtained by deleting the *spmA* gene, which is responsible for the metabolism of SP into HSP, by homologous recombination. The study demonstrated the efficiency of the process through two distinct routes: strategy A, which uses a purified nicotine solution from TW to obtain a high-purity product with a crystal isolation yield of 54.2% relative to the initial nicotine content, and strategy B, which directly employs a raw tobacco suspension. The latter is considered a more environmentally friendly method as it eliminates the use of toxic organic solvents such as chloroform, relying instead on a simple water extraction at 60°C. Results obtained under fed-batch conditions showed titers of 9.8 g/L SP for strategy A and 8.9 g/L for strategy B, demonstrating that the process can be efficient even in complex media. A critical aspect of the optimization was pH control. While the optimal pH for pure nicotine was 9.0, maintaining a pH of 7.0 was necessary for the raw extract to

prevent inhibition of the biocatalyst by complex tobacco components such as pectins and tannins. The whole-cell biocatalyst demonstrated remarkable stability, being successfully reused over three consecutive batch cycles with conversion yields of 97.0%, 100.0%, and 87.9%, respectively. This study highlights the industrial potential of *Pseudomonas* strain as a robust cell factory capable of recycling TW and converting it into valuable precursors for vitamin B6, anti-hypertensive drugs, and analgesics such as epibatidine (10).

***Agrobacterium tumefaciens* strains**

Research on the *A. tumefaciens* S33 strain focuses on the valorization of TW through the biotransformation of nicotine into high-value pyridine compounds.

The first study details an environmentally friendly route for the production of 6HN by blocking nicotine catabolism at this intermediate level, a process achieved by identifying and disrupting the *hno* gene that encodes the enzyme 6-hydroxynicotine oxidase, which catalyzes the oxidation of 6HN into 6-hydroxy-N-methylmyosmine (6NMM). This genetic modification, performed via homologous recombination using a suicide plasmid (pJQ200SK), generated the mutant strain S33- Δ *hno*, which lost its ability to further metabolize 6HN. In this case, whole mutant cells were used as a biocatalyst, a method applied exactly as with the previously described *Pseudomonas* strains. Under optimal conditions of 30°C and pH 7.0, the researchers achieved a molar conversion rate of approximately 98% and a specific catalytic rate of 1.01 g of product per hour per gram of dry cells. Under fed-batch conditions, the bioconversion of 4 g/L nicotine to 4.70 g/L 6HN was achieved. The final product was extracted with dichloromethane, yielding a recovery rate of 76.8% and serving as an essential precursor for the development of drugs for Alzheimer's and Parkinson's diseases (12).

The second study provides additional insight into the biochemical mechanism by identifying and characterizing Ald, an NAD-specific aldehyde dehydrogenase that was the last uncharacterized enzyme in the hybrid nicotine degradation pathway (VPP) in the *A. tumefaciens* S33 strain. This enzyme is involved in the conversion of 6-hydroxy-3-succinoyl-semialdehyde-pyridine to HSP. Its function was confirmed both by *in vitro* enzymatic assays using the SAP analog and by the deletion of the *ald* gene, which reduced the growth rate and biomass of the bacterium on nicotine-containing media. In contrast to similar enzymes in other strains that utilize NADP, Ald from the *A. tumefaciens* S33 strain is strictly NAD-dependent and exhibits remarkable versatility, possessing a broad substrate spectrum. In addition to nicotine metabolism intermediates, such as HSP, Ald can also efficiently metabolize other substances such as benzaldehyde, furfural, and acetaldehyde, exhibiting optimal activity at a pH of 9.0. To explore this potential, the *ald* gene was heterologously introduced into *E. coli* BL21 and DH5 α strains, yielding recombinant variants such as Ald-N (equipped with a His tag at the N-terminus, proving to be more stable and active) and Ald-C (with a His tag at the C-terminus). The biotechnological application of this enzyme has been expanded through the use of recombinant *E. coli* cells expressing the *ald* gene, known as *E. coli*_Ald, which function as highly efficient biocatalysts. Using these, the efficient conversion of furfural to 2-furoic acid was achieved with a yield of 95.4% in just 2 hours, reaching 99% after 10 hours of reaction. These findings not only fully elucidate electron transfer and redox metabolism in nicotine degradation in *Agrobacterium*, but also provide new tools for the industrial catalysis of bio-based furan compounds used in the production of plastics and pharmaceuticals, a precursor for antibiotics, anti-hypertensive, and anti-parasitic drugs, thereby integrating toxic TW into a circular economy of renewable resources (11).

Novel NDM applications

Recent research has established that *Enterobacter hormaechei* ZS01, a bacterium isolated from cherry red tobacco, represents a new NDM strain identified for the first time with this capability, being able to efficiently catabolize nicotine through the simultaneous activation of the pyrrolidine pathway and a demethylation pathway. The strain was genetically modified to

improve SP production, starting with the deletion of the *moaE* gene, involved in the biosynthesis of the molybdenum cofactor, to inactivate SpmABC monooxygenase, responsible for the conversion of SP to HSP, which generated the mutant strain ZS1 with a 2.78-fold higher accumulation of SP compared to the wild-type strain. Subsequently, membrane permeability was optimized to facilitate nicotine absorption and SP release. This was achieved by modifying the structure of lipopolysaccharides through the deletion of the *rfaF* and *rfaC* genes, which encode heptosyltransferases responsible for the assembly of the core oligosaccharide layer. This genetic engineering strategy resulted in the ZS5 strain, which reached the highest SP concentration of 1.42 g/L in flask, while the triple mutant ZS8 ($\Delta rfaC\Delta rfaF\Delta lpxM$), which further incorporates the deletion of the *lpxM* gene encoding a myristoyl transferase involved in lipid A biosynthesis, exhibited a 3.60-fold higher permeability but demonstrated a low yield due to the excessive disruption of cellular physiological functions and energy metabolism. In terms of yield, strain ZS1 demonstrated a nicotine conversion rate of 98.01% at concentrations of 1 g/L in liquid media and 95.80% in the TWE after 48 hours, maintaining remarkable tolerance at concentrations up to 5 g/L and adaptability across wide temperature (28 - 40°C) and pH (5.5 - 8.5) ranges. Maximum performance was achieved in a 5 L bioreactor, where the use of strain ZS5 in fed-batch mode generated an SP titer of 4.11 g/L with a conversion rate of 68.82%, exceeding the previous values of 3.3 g/L reported for other NDMs. The biotechnological applications of this approach are significant, as the strain functions as a cellular factory that converts TW into precursors for anti-hypertensive drugs, compounds with anti-cancer, analgesic, and neuroprotective properties such as acetophenone (whose content increased by 391.20%), valuable substances for cosmetics, pharmaceuticals, and agriculture such as neophytadiene (98.78% increase), and monomers used in the manufacture of high-strength plastics and rubbers such as α -methylstyrene (an increase of 97.37%), thus providing a sustainable solution for the ecological management of TW (15, 16).

Nicotine-tolerant non-NDMs

Research shows that some non-NDM strains possess remarkable resistance to nicotine, making them great candidates for producing valuable bioactive compounds.

An example of this microorganism is *Bacillus amyloliquefaciens* T4, an osmotolerant bacterium able to grow in the presence of nicotine and high osmotic pressure. Additionally, this microorganism can use TW as a nitrogen co-substrate to produce 2,3-butanediol (2,3-BD), a compound that has potential applications in the renewable fuel industry and in the production of bioplastics. The approach involves cellular biocatalysis in submerged fermentation at 37°C and pH 7.0, yielding 1.54 g/L of 2,3-BD by using the soluble sugars naturally contained in TWE ($\approx 3.07\%$) as a carbon source, while nicotine is tolerated and potentially utilized as growth factor, achieving yields of 30.06 g/L at 48 hours and 42.21 g/L at 72 hours when the process is supplemented with 200 g/L glucose as the primary carbon source and 50 g/L TWE as the nutrient source (17).

Similarly, the *Acetobacter xylinum* ATCC 23767 strain was used to produce bacterial cellulose (BC) using TWE as a substrate, which contains 25.1% sugars (glucose, fructose, sucrose) and 2.2% nicotine. Nicotine was removed from TW via distillation at pH 9.0, achieving a recovery rate of 88.7% - 90% without degrading the sugars necessary for BC synthesis. Implementing a two-stage fermentation over 16 days was essential: the first 7-day stage produced 2.27 g/L BC, but the decline in pH to 2.65 due to acid metabolism halted synthesis, even though more than half of the carbon sources remained unused. By harvesting the initial film to remove the oxygen transfer limitation and readjusting the pH to 6.5, the process was resumed, reaching a final yield of 5.2 g/L BC. BC is a biopolymer composed of glucose units joined by β -1,4-glycosidic bonds. It features a dense network of ultrafine fibers with properties superior to those of plant cellulose, including high purity, tensile strength, porosity, high water retention capacity, and thermal

stability up to 250°C. Due to its biological adaptability and mechanical characteristics, BC obtained from TW has wide-ranging applications, particularly in the textile industry (18).

Another approach focuses on the *Sinorhizobium meliloti* 224 strain, a symbiotic soil bacterium identified for its ability to produce cellulases (Avicelase and CMCase) through the bioconversion of TW. This bacterium was used both in submerged fermentation (SmF) and solid-state fermentation (SSF) conditions. In SmF, the optimal conditions were established at a concentration of 5 g/L of TW, an incubation time of 2 days, and a 9% inoculum, while the strain was optimized through acclimatization and successive passages on carboxymethyl cellulose media to promote metabolic adaptation, thereby achieving a roughly 3-fold increase in enzymatic activity. By chemically modifying the substrate with 1% NaOH, which increases the internal surface area and reduces the degree of polymerization and crystallinity of the lignocellulose, maximum yields of 1.503 U/g (Avicelase) and 1.615 U/g (CMCase) were achieved in SSF using a 10% acclimatized inoculum. These industrial enzymes, in which exoglucanase activity (Avicelase) is predominant, are fundamental for the bioconversion of TW rich in lignocellulosic material into useful products, with major applications in the textile, bioethanol, and animal feed industries (19).

At the end, the study on the *Bacillus subtilis* 168 strain demonstrates the transformation of a safe GRAS-certified microorganism (Generally Recognized as Safe) into an effective nicotine-removal system through the use of CRISPR-Cas9 genome editing technology. To create a functional metabolic pathway, the researchers integrated the nicotine oxidoreductase variant (*NicA2v*) and pseudooxynicotine amine oxidase (*Pppnao-S16*) genes – both derived from *P. putida* S16 – into the host genome to sequentially convert nicotine into SAP. To ensure cell viability and mitigate the metabolic stress caused by competition for FAD cofactors, the constitutive P43 promoter, which allows stable and controlled expression of the first enzyme. To manage the high toxicity of the second enzyme, a novel uORF translational attenuator (*KppqqA*), acting as a fine-tuning switch that limits protein production to an optimal level without compromising bacterial viability. System efficiency was further enhanced by introducing the *R54Q* mutation into the distal loop of the *Pppnao-S16* gene, which increased the reaction rate of the enzyme by 30% without altering substrate affinity, while the strategic integration of these genetic cassettes at the *LacA*, *LytC*, and *LytD* loci allowed for the simultaneous disruption of native autolysis genes, thereby significantly improving late-stationary-phase robustness and culture stability over time. The resulting strain, designated Bs.3.0, demonstrated the ability to degrade over 99% of nicotine within 24 hours and showed a 10-fold higher tolerance to this alkaloid compared to the wild-type strain. This system has key applications in the bioremediation of water, and soil, as well as in the development of probiotics for *in vivo* detoxification, protecting human health by preventing the formation of nicotine-derived carcinogenic compounds (20).

Co-culture systems

Recent research proposes an innovative approach to the valorization of TW by using co-culture systems, in which NDMs and non-NDMs are grown simultaneously to enhance environmental detoxification through the production of valuable metabolites, thereby overcoming the limitations of pure cultures where nicotine drastically inhibits the growth of productive microorganisms.

A highly efficient cellular method involves using the *Acetobacter oryzoeni* MGC-N8819 strain in co-culture with *Pseudomonas* sp. JY-Q/5Δ, an approach that eliminates the need for complex physico-chemical treatments to remove nicotine. In this system, the *Pseudomonas* strain was genetically modified by deleting five genes essential for the first step of glucose metabolism, transforming it into a selective NDM without consuming the glucose required by the first strain, thereby avoiding competition for the carbon substrate. The production yield for BC increased significantly in this co-culture system, reaching 4.61 g/L after 7 days, compared to only 3.27

g/L in the pure culture, and by applying a two-stage fermentation strategy, the final yield reached 6.00 g/L BC. The biotechnological applications of this cellulose are vast, the material obtained exhibits thermal stability and superior mechanical properties, making it applicable in the textile industry (21).

Another advanced system of microbial co-culture consists in two strains: *Arthrobacter nicotinovorans*, and a metabolically engineered strain of *E. coli*, with the aim of achieving multi-metabolic conversion of tobacco dust. The method used is integrated cellular bioconversion, where the division of metabolic steps is clearly distributed: *A. nicotinovorans* ensures rapid detoxification of nicotine (approximately 430 mg/L) within 96 hours via the pyridine pathway, thereby protecting the sensitive strain of *E. coli*. The *E. coli* tMTScLS strain was genetically modified by co-transformation of two plasmids (pMVA1 and pScLS) containing genes for the mevalonate pathway and linalool synthesis – *Scmk* (mevalonate kinase), *Scpmk* (phosphomevalonate kinase), and *Scpmd* (mevalonate pyrophosphate decarboxylase) from *Saccharomyces cerevisiae*, and *Sahmgs* (HMG-CoA synthase) and *Sahmgr* (HMG-CoA reductase) from *Staphylococcus aureus*. The yield of this integrated system, optimized at an inoculation ratio of 3:1, allowed for the production of 8.3 g/L ethanol and 0.17 g/L linalool directly from the tobacco dust, demonstrating a synergy where the nicotine detoxification performed by *Arthrobacter* facilitates sugar metabolism and monoterpene synthesis by *E. coli* tMTScLS. The applications of this system are directly aimed at the circular bioeconomy through the sustainable production of ethanol, used as a biofuel, and high-value aromatic compounds, like linalool, which are widely used in the cosmetics, and pharmaceutical industries, providing a modular platform that can be adapted to produce other types of terpenoids or industrial flavors (22).

Discussions and Conclusions

This systematic review confirms that the microbial valorization of TW is a practical and promising way to support a circular economy. Most research focuses on two types of NDMs, *P. putida* and *A. tumefaciens*, which are representative strains for the pyrrolidine and VPP pathways, turning nicotine from TW into valuable products like functional pyridines. Interest in this field has grown significantly since 2019, following the discovery of new NDMs with extended biotechnological applications and the development of co-culture systems for the valorization of TW. At the same time, genome editing tools, such as CRISPR-Cas9, have made it possible to confer a new metabolic property related to nicotine degradation, thereby opening the road to applications that offer new perspectives for research.

Moving from the lab to a factory setting is the real hurdle. While these processes work in small flasks, scaling up to an industrial level is much more difficult. Because each lab uses different experimental conditions, it is extremely hard to establish a common protocol for a specific species to both decontaminate nicotine and produce valuable bioactive compounds at the same time. Additionally, compounds meant for medical use need to be tested outside the lab and validated through *in vivo* trials on animals or humans to prove they are safe and non-toxic.

The present systematic review is subject to several methodological limitations that must be acknowledged. First, the screening and data extraction process was conducted by a single author, which introduces a potential risk of bias in both study selection and interpretation. Furthermore, the exclusive inclusion of articles published in English since 2014 with an impact factor of $IF \geq 1.5$ may have led to the exclusion of valuable research published in other languages, in lower-visibility journals, or prior to this timeframe. Additionally, due to the high degree of methodological heterogeneity regarding experimental conditions and bacterial strains, it was not possible to perform a quantitative meta-analysis. Consequently, a narrative synthesis approach was adopted, which is inherently more subjective in its nature.

The findings of this review carry significant implications for research, industrial practice, and public policy. Scientific efforts should focus on the transition of biotechnological processes from the laboratory to an industrial level while ensuring the safety and quality of the obtained compounds. In the industrial sector, the application of these biotechnological methods could reduce environmental impact and create economic value from TW. Finally, the adoption of regulations that incentivize the valorization of industrial TW, in accordance with the 2030 Agenda and circular economy principles, could facilitate the extensive implementation of these sustainable solutions.

Declarations

Funding: This review received no external support;

Competing interests: The author declares none

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Appendix 1 – PRISMA 2020 Checklist

Section and Topic	Item#	Checklist item	Location where item is reported ⁽¹⁾
TITLE			
Title	1	Identify the report as a systematic review.	1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	1-2
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	1-2
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	3
Information sources	6	Specify all databases, registers, websites, organizations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	2
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	2-3
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	3
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	3
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	3
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	N/A ¹
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	3
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	N/A ¹
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	3
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	N/A ¹
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	N/A ¹

Section and Topic	Item#	Checklist item	Location where item is reported ^(*)
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	N/A ¹
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	N/A ¹
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	N/A ¹
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	N/A ¹
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	N/A ¹
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	4-5
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	4-5
Study characteristics	17	Cite each included study and present its characteristics.	4-5
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	N/A ¹
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimates and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	5-10
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	5-10
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	N/A ¹
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	N/A ¹
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	N/A ¹
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	N/A ¹
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	N/A ¹
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	10-11
	23b	Discuss any limitations of the evidence included in the review.	10-11
	23c	Discuss any limitations of the review processes used.	10-11
	23d	Discuss implications of the results for practice, policy, and future research.	10-11

Section and Topic	Item#	Checklist item	Location where item is reported ^(*)
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	N/A ¹
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	N/A ¹
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	N/A ¹
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	11
Competing interests	26	Declare any competing interests of review authors.	11
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Appendix 2

*the pages where the checklist items are located; ¹item applicable for meta-analysis.

Appendix 2 – Comprehensive overview of the included studies

Nr.	Author	Title	Year	Journal	Bacterial strain	Substrate	Bioactive Compound	Application
1	Shang <i>et al.</i>	An NAD-Specific 6-Hydroxy-3-Succinoyl-Semialdehyde-Pyridine Dehydrogenase from Nicotine-Degrading <i>Agrobacterium tumefaciens</i> Strain S33	2021	Microbiology Spectrum	<i>Agrobacterium tumefaciens</i> S33 and <i>E. coli</i> _Ald	Nicotine and furfural	6-hydroxy-3-succinoyl-pyridine and 2-furoic acid	Industrial biocatalysis; Green chemistry; Pharmaceutical and plastics industries
2	Lei <i>et al.</i>	Valorization of tobacco waste leaves: Discovery of <i>Enterobacter hormaechei</i> ZSS01 for efficient nicotine degradation	2025	Biochemical and Biophysical Research Communications	<i>Enterobacter hormaechei</i> ZSS01	Nicotine from tobacco waste leaves	Acetophenone; Neophytadiene; α -methylstyrene	Food, cosmetics, pharmaceutical, and materials industries
3	Ye <i>et al.</i>	Bacterial cellulose production by <i>Acetobacter xylinum</i> ATCC 23767 using tobacco waste extracts as culture medium	2019	Bioresource Technology	<i>Acetobacter xylinum</i> ATCC 23767	Tobacco Waste Extracts	Bacterial cellulose	Textile industry
4	Wang <i>et al.</i>	Sustainable production of valuable compound 3-succinoyl-pyridine by genetically engineering <i>Pseudomonas putida</i> using the tobacco waste	2015	Scientific Reports	<i>Pseudomonas putida</i> S16 Δ spm	Tobacco Waste	3-succinoyl-pyridine	Pharmaceutical industry
5	Yu <i>et al.</i>	Green strategy from waste to value-added-chemical production: efficient biosynthesis of 6-hydroxy-3-succinoyl-pyridine by an engineered biocatalyst	2014	Scientific Reports	<i>Pseudomonas putida</i> P-HSP	Tobacco Waste Extracts	6-hydroxy-3-succinoyl-pyridine	Industrial biocatalysis; Environmental protection and bioremediation; Pharmaceutical industry
6	Nie <i>et al.</i>	Improved bacterial cellulose production by <i>Acetobacter oryzoeni</i> MCG-N8819 in tobacco waste extract coupled with nicotine removal by <i>Pseudomonas</i> sp. JY-Q-5 Δ	2025	International Journal of Biological Macromolecules	<i>Acetobacter oryzoeni</i> MCG-N8819 and <i>Pseudomonas</i> sp. JY-Q-5 Δ	Tobacco Waste Extracts	Bacterial cellulose	Textile industry
7	Han <i>et al.</i>	Isolation and identification of an osmotolerant <i>Bacillus amyloliquefaciens</i> strain T4 for 2,3-butanediol production with tobacco waste	2022	Preparative Biochemistry and Biotechnology	<i>Bacillus amyloliquefaciens</i> T4	Tobacco Waste Extracts	2,3-butanediol	Pharmacy; Green chemistry; Agriculture; Industrial biocatalysis; Bioremediation

Nr.	Author	Title	Year	Journal	Bacterial strain	Substrate	Bioactive Compound	Application
8	Zhang <i>et al.</i>	Integrated microbial co-culture engineering for sustainable valorization of tobacco waste	2026	Journal of ecological Engineering	<i>Arthrobacter nicotinovorans</i> and <i>E. coli</i> tMTScLS	Tobacco Dust	Ethanol and linalool	Biofuel; Pharmaceutical and cosmetic industries
9	Buntic <i>et al.</i>	Cellulase production by <i>Sinorhizobium meliloti</i> strain 224 using waste tobacco as substrate	2019	International Journal of Environmental Science and Technology	<i>Sinorhizobium meliloti</i> strain 224	Tobacco Waste	Cellulase	Food, Biofuel and textile industries
10	Lei <i>et al.</i>	Targeted membrane permeability alterations in <i>Enterobacter hormaechei</i> ZS01 for high-level production of 3-succinoyl-pyridine from tobacco waste	2025	World Journal of Microbiology and Biotechnology	<i>Enterobacter hormaechei</i> ZS01	Tobacco Waste	3-succinoyl-pyridine	Industrial biotransformation; Agrochemical manufacturing; Pharmaceutical industry
11	Wang <i>et al.</i>	Proximity-enhanced co-immobilized enzyme cascade for efficient bioconversion of nicotine to 3-succinoylpyridine	2026	Bioresource and Bioprocessing	<i>Pseudomonas putida</i>	Tobacco Waste	3-succinoyl-pyridine	Pharmaceutical industry
12	Yu <i>et al.</i>	Green route to synthesis of valuable chemical 6-hydroxynicotine from nicotine in tobacco wastes using genetically engineered <i>Agrobacterium tumefaciens</i> S33	2017	Biotechnology for Biofuels	<i>Agrobacterium tumefaciens</i> S33	Tobacco Waste	6-hydroxynicotine	Pharmaceutical industry
13	Li <i>et al.</i>	Synergic engineering of a GRAS-certified strain: Transforming <i>Bacillus subtilis</i> 168 into a high-performance nicotine scavenger	2026	Journal of Hazardous Materials	<i>Bacillus subtilis</i> 168	Nicotine	3-succinoyl-semialdehyde-pyridine	Environmental protection and bioremediation; Nicotine probiotic scavenger for human health

