SHORT REPORT

ACG publications

Rec. Nat. Prod. 18:5 (2024) 532-537

records of natural products

Evaluation of Essential Oil Components in Genetically Modified Poaceae Plants: A Comparative Study of Their Whitening and Antioxidant Activities In Vitro

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(Received April 23, 2024; Revised August 06, 2024; Accepted August 07, 2024)

Abstract: This study aimed to modify the essential oils of *Cymbopogon flexuosus* (Nees ex Steud.) W.Watson, hereafter referred to as CF, and *Cymbopogon martini* (Roxb.) W.Watson, hereafter referred to as CM, utilizing an acid-distillation protocol. The modification resulted in a significant reduction in the aldehyde components, Neral and Geranial, in CF, subsequently leading to the formation of the monoterpene, Cymene. Conversely, the alteration in CM decreased the proportions of the monoterpenol, Geraniol, and the ester, Geranyl acetate. These modifications enhanced the cellular safety of both CF and CM essential oils towards B16-F10 cells in vitro, increasing it by 4 to 8-fold, and concurrently improved their melanin suppression and antioxidant properties.

Keywords: Essential oil safety; acid-distilled; whitening; antioxidant..

1. Plant Source

In September 2020, seedlings of CF and CM were acquired from the Taiwan Forestry Research Institute and subsequently cultivated at an elevation of 500 meters in Guoxing Township, Taiwan. The CF specimen has been catalogued with the accession code E Tropicos 101165511; conversely, the CM specimen is recorded under the accession code BM Tropicos 101165537 and is preserved within the Tropicos Specimens Non-MO collection.

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The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products September-October 2024 EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.473.2404.3209
Available online: September 03, 2024

2. Previous Studies

Previous research employing an acid-distillation protocol demonstrated a reduction in citral concentration from 72.60% to 30.07% in lemongrass extracts [1]. This reduction is speculated to potentially enhance cellular safety. Consequently, the present study adapts this acid-distillation protocol to CF and CM, both members of the Poaceae family, to assess alterations in the survival of B16F-10 cells, melanin suppression, and antioxidant properties.

3. Present Study

Chemicals: B16-F10 melanoma cells were obtained from the Bioresource Collection and Research Center (BCRC) in East Dist, HSZ, TWN. All other chemicals used, including 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), were of analytical reagent grade and sourced from ECHO CHEMICAL CO., LTD., Toufen City, ZMI, TWN. Ultrapure water was utilized throughout the analytical procedures.

Modified Extraction Method: One hundred kilograms each of CF and CM were freshly harvested and subjected to steam distillation as the initial extraction process, yielding essential oils referred to as CF Stock Solution (CFSS) and CM Stock Solution (CMSS), respectively. Following a modified method by Ajayi et al. [1], 100 mL of either CFSS or CMSS was added to an aqueous solution containing 5% citric acid and stirred. A secondary distillation using water distillation was then performed to produce CF Acidic (CFAC) and CM Acidic (CMAC) test samples. Similarly, monoterpenes Citral and Geraniol were processed as Citral Stock Solution (CalSS) and Geraniol Stock Solution (GolSS) to obtain Citral Acidic (CalAC) and Geraniol Acidic (GolAC) test samples.

Gas Chromatography Mass Spectrometry (GC-MS): Following modifications described by Adams [2], the analysis was conducted using a GC7890 gas chromatograph coupled with a 5977B MSD quadrupole mass spectrometer. The analytical column was a DB-5 MS capillary column, 30 m in length, with an internal diameter of 0.25 mm and a film thickness of 0.25 µm (Agilent Technologies, Inc., Santa Clara, CA, USA). The injection was performed in split mode at a constant temperature of 300°C, and helium gas of ≥99.999% purity was used as the carrier gas at a flow rate of 1 mL/min. The temperature program increased from 50°C to 280°C at a rate of 3°C per minute, followed by a 5-minute isothermal hold. The mass spectrometer operated at 70 eV under Electron-impact Ionization (EI). The spectral data obtained were analyzed using the National Institute of Standards and Technology (NIST) database. Additionally, the retention times of the analytes and an Alkane standard solution (C_8-C_{20}) were collected to calculate the Kovats Index (KI), which was compared against values reported in Adams publication, using standards for reference.

In-vitro Cytotoxicity Assay: Improving methodology according to Poulose et al. [3]. Cells were seeded at a density of 6,000 per well in a 96-well plate, with each well receiving 100 µL of culture medium and incubated for 24 hours. After aspiration of the Dulbecco's Modified Eagle Medium (DMEM), the cells were treated with various concentrations of the test samples for another 24 hours before the medium was removed. The cells were then washed with 150 µL/well of Phosphate Buffered Saline (PBS). Subsequently, 100 µL of MTT reagent (0.5 mg/mL) was added to each well. The plates were incubated in the dark in a CO2 incubator for 30 minutes before the MTT reagent was aspirated. Following a PBS wash, 100 µL/well of Dimethyl Sulfoxide (DMSO) was added to dissolve the formazan crystals. The solution was mixed thoroughly, and the absorbance at 540 nm was measured using an ELISA reader.

Melanin Content: Improving methodology according to Chatatikun et al. [4]. B16-F10 cells were seeded at a density of 2 × 10^5 cells per well in 6-well plates and cultured at 37°C in a 5% CO2 incubator for 24 hours. Subsequent to this initial incubation, various concentrations of test samples were added, and the cells were cultured for an additional 24 hours. The medium was then washed off with Phosphate Buffered Saline (PBS), and an appropriate amount of trypsin was added to detach the

cells. The cells were centrifuged at 4,500 rpm for 15 minutes, and the supernatant was discarded. To extract melanin, 120 µL of 1N NaOH was added to the cell pellets and the mixture was incubated in a 65°C water bath for one hour. After cooling to room temperature, 100 µL of the solution was transferred to a 96-well plate, and the absorbance at 405 nm was measured using an ELISA reader.

ABTS Radical Scavenging Assay: Serial dilutions were prepared according to the safety concentration for B16-F10 cells, with improvements described by in the former studies [5,6]. A volume of 0.1 mL of ethanol was combined with 0.02 mL of either ABTS ethanol solution or ethanol alone, followed by the addition of 0.28 mL of the sample. The solution was shaken and then incubated in the dark at room temperature for 10 minutes. Absorbance was measured at 734 nm using an ELISA reader, and the scavenging efficiency (%) was calculated.

DPPH Radical Scavenging Assay: Dilutions aligned with the B16-F10 cells safe dosage sequence were meticulously prepared, incorporating refinements as delineated in the literature [6-9]. A total of 0.1 mL of 100 mM Tris-HCl buffer was combined with 0.02 mL of either 2.5 mM DPPH solution or ethanol. Subsequently, 0.28 mL of the sample was added, resulting in a final DPPH concentration of 0.125 mM. The mixture was shaken and then allowed to stand in the dark at room temperature for 30 minutes. The absorbance at 517 nm was measured using an ELISA reader, and the scavenging efficiency (%) was calculated.

Identification of CF, Citral CM, and Geraniol Modified Extractions by GC-MS: Refer to Figure 1 the essential oil color of CFSS is a brown hue, while CFAC essential oil is a light yellow color. Refer to Table S1 CFSS was found to contain 13 compounds, with Neral at 30.54% and Geranial at 38.14% being the principal components. The analogs in CFSS are monoterpenes at 2.08%, sesquiterpenes at 9.02%, monoterpenols at 4.26%, phenols at 1.52%, aldehydes at 69.89%, esters at 7.41%, ketones at 3.16%, and other oxides at 1.41% (Table S2). CFAC was identified to contain 26 compounds, with primary components being Neral at 14.84%, Geranial at 20.02%, and *o*-Cymene at 12.08% (Table S1). The results are consistent with previous studies by Ajayi et al. [1]. The analogs in CFAC include monoterpenes at 22.85%, sesquiterpenes at 8.13%, monoterpenols at 18.59%, phenols at 0.82%, aldehydes at 37%, esters at 5.71%, ketones at 2.59%, and other oxides at 2.32% (Table S3).

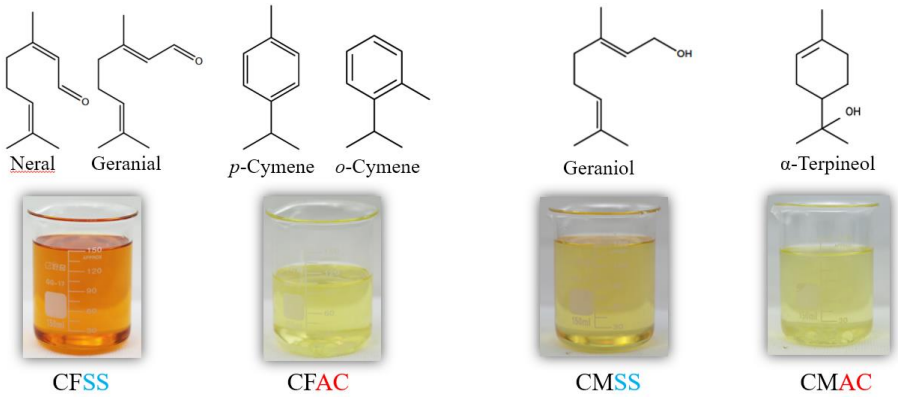

Figure 1: Photographs and Representative Components of CM and CF Essential Oils

For citral analysis, please refer to Table S1 CalSS was analyzed and found to consist of two compounds, Neral (51.71%) and Geranial (48.29%), collectively representing 100% aldehydes. Conversely, CalAC encompassed nine compounds, with predominant constituents identified as Geranial (26.97%), Neral (23.61%), *o*-Cymene (15.56%), δ-Terpineol (10.76%), *p*-Cymene (8.67%), and α-Terpineol (6.23%). It has been established that the compounds Cymene, δ-Terpineol, and α-Terpineol in both CFAC and CalAC originated from the modifications applied to CFSS and CalSS. Following modification, the aldehyde proportion in citral was reduced from 100% to 52.64%, resulting in the formation of monoterpenes (26.21%) and monoterpenols (16.99%). The experimental outcomes for CFAC and CalAC were found to be consistent, as detailed in Tables S3 and S4.

Please refer to Figure 1 for visual distinctions CMSS essential oil is characterized by a deeper yellow hue, whereas CMAC oil is noted for its lighter yellow shade. Analysis documented in Table S5 revealed that CMSS comprises seven compounds, with Geraniol (54.27%) and Geranyl acetate (20.93%) as the predominant components. The constituent profile of CMSS includes monoterpenes (3.54%), sesquiterpenes (5.92%), monoterpenols (60.4%), sesquiterpenols (3.61%), aldehydes (2.15%), and esters (20.93%), as detailed in Table S6. Conversely, CMAC is composed of 26 identified compounds, with Geraniol (17.2%), α-Terpineol (17.12%), and Geranyl acetate (9.96%) being the principal components, as reported in Table S5. The analog spectrum within CMAC includes monoterpenes (12.48%), sesquiterpenes (3%), monoterpenols (64.2%), sesquiterpenols (0.68%), esters (9.96%), and other oxides (8.34%), as cataloged in Table S7.

Refer to Table S5 for the analysis of Geraniol. GolSS was found to predominantly contain Geraniol, representing 95.09% of monoterpenols. GolAC was determined to encompass 21 compounds, with the principal constituents identified as α-Terpineol (22.14%), Linalool (14.16%), Geranic oxide (6.36%), cis-Ocimenol (6.79%), and Terpinolene (5.54%). It has been established that the compounds α-Terpineol, Linalool, Geranic oxide, cis-Ocimenol, and Terpinolene present in CMAC and GolAC originated from the modifications of CMSS and GolSS. Following these modifications, the content of monoterpenols in Geraniol was reduced from 95.09% to 73.73%, resulting in the formation of monoterpenes (14.28%) and other oxides (11.95%). Consistent experimental outcomes for CMAC and GolAC are detailed in Tables S7 and S8.

In our GC-MS analyses, all constituents were systematically categorized into analogs to streamline the organization of complex components. Notably, components within the monoterpenol group were identified for their anti-inflammatory properties, whereas those in the ester group were found to contribute to elastin synthesis, thus exhibiting anti-wrinkle effects [10-13]. Given that analogous structural activities often correlate with similar functions, future research will focus on classifying these analogs to reveal common features across different essential oils under complex compositional conditions.

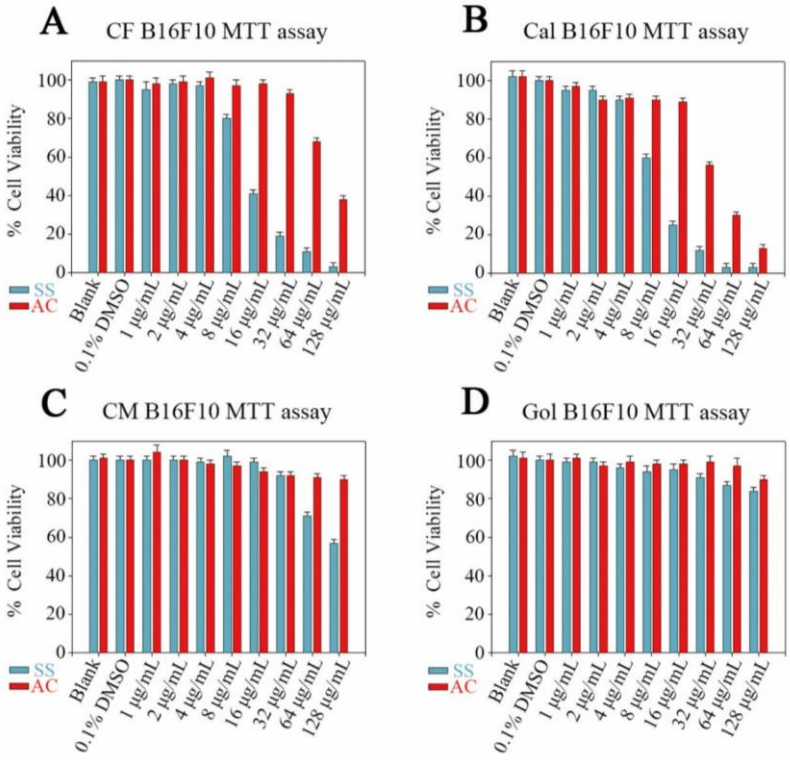

Figure 2. The impact of CFSS and CFAC (A), CalSS and CalAC (B), CMSS and CMAC (C), as well as GolSS and GolAC (D) on the viability of B16F10 melanoma cells after 24 hours of exposure. Results are presented as mean ± standard deviation (n = 3).

CytotoxicityAassay of CF, Citral, CM and Geraniol in B16-F10 Cells in Vitro: Refer to Figure 2A for the CF analysis. All cell survival rates were established at a minimum of 90% to define safe dosages. The safety range for CFSS was determined to be from 1 μg/mL to 4 μg/mL. For the modified CFAC, the safety range extended from 1 μg/mL to 32 μg/mL, an 8-fold increase in safety relative to CFSS. This improvement is attributed to the decreased proportions of Neral and Geranial, a reduction in aldehydes, and increases in the concentrations of monoterpenes and monoterpenols (Tables S1-S3). Regarding Citral, as depicted in Figure 2B, the safe dosage for CalSS was established between 1 μg/mL and 4 μg/mL, while for CalAC, it extended to 16 μg/mL, indicating a 4-fold enhancement in safety. For the CM analysis in Figure 2C, the safety dosage for CMSS was found to be between 1 μg/mL and 32 μg/mL, and for CMAC, it expanded to 128 μg/mL, reflecting a 4-fold increase. For Geraniol, detailed in Figure 2D, GolSS maintained a cell survival rate of 85% at 64 μg/mL, defining a safe dosage range from 1 μg/mL to 32 μg/mL. For GolAC, the range extended to 128 μg/mL, aligning with the outcomes observed for Geraniol and CM.

Melanogenesis Assay in B16-F10 Melanoma Cells of CF Citral CM and Geraniol: Refer to Figure S1A. At a maximum safe dosage of 4 μg/mL, CFSS exhibited a melanin content of 80%, corresponding to a 20% melanin inhibition. In contrast, CFAC, at its upper safety limit of 32 μg/mL, demonstrated a melanin content reduction to 60%, which equates to a 40% inhibition, effectively doubling the inhibitory efficacy observed with CFSS. Turning to Citral, depicted in Figure S1B, CalSS maintained a melanin content of 80% at a maximum dosage of 4 μg/mL, achieving a 20% inhibition. CalAC, at a dosage ceiling of 16 μg/mL, displayed a melanin content of 82%, with an inhibition rate of 18%. Notably, CFAC exhibited twice the melanin inhibition efficiency of CalAC at their respective maximum safe dosages. For CM, detailed in Figure S1C, CMSS, at a dosage of 32 μg/mL, recorded a melanin content of 73%, inhibiting by 27%. CMAC, at 128 μg/mL, showed a melanin content of 75%, achieving a 25% inhibition. Lastly, for Geraniol, as shown in Figure S1D, GolSS at a dosage of 32 μg/mL held a melanin content of 85%, with a 15% inhibition, whereas GolAC, at 128 μg/mL, reached a melanin content of 83%, inhibiting by 17%.

Antioxidant Activity Analysis of CF and CM on ABTS and DPPH Free Radicals In Vitro: Refer to Figure S2A for the analysis of CF. At its maximum permissible concentration of 4 μg/mL, CFSS was found to clear 56.17% of ABTS radicals, with an IC_{50} value of 3.64 μg/mL, affirming its efficacy in scavenging ABTS radicals. Conversely, CFAC, at a maximum permissible concentration of 32 μg/mL, cleared 98.26% of ABTS radicals, with an IC_{50} value of 3.59 μg/mL. This represents a 42.09% increase in scavenging efficiency over CFSS at comparable safe dosages. Refer to Figure S2B for the CM analysis. At its maximum safe concentration of 32 μg/mL, CMSS cleared 81.22% of ABTS radicals, with an IC_{50} of 11.73 μg/mL. CMAC, at a higher safe concentration of 128 μg/mL, was able to clear 98.21% of ABTS radicals, with an IC_{50} of 12.49 μg/mL, demonstrating a 16.99% increase in scavenging efficiency over CMSS at their respective safe concentrations.

Refer to Figure S3A for CF analysis. CFSS, at its maximum safe concentration of 4 μg/mL, exhibited an 82.14% clearance of DPPH free radicals; the IC_{50} was determined to be 1.41 μg/mL. In parallel, CFAC at the same concentration cleared 81.22% of DPPH radicals, with an IC_{50} of 1.78 μg/mL, indicating consistent results between CFSS and CFAC. Refer to Figure S3B for CM data. CMSS showed an IC_{50} of 46.53 μg/mL. Notably, concentrations exceeding the cellular safety threshold of 32 μg/mL may induce cytotoxic effects. Conversely, CMAC at a maximum safe dosage of 128 μg/mL was capable of clearing 58.42% of DPPH radicals, with an IC_{50} of 62.16 μg/mL, effectively demonstrating its antioxidant efficacy within the established safe dosage parameters.

This preliminary in vitro study observed that modifications through an acid-distilled program could enhance the safety and increase the inhibitory effect on melanogenesis and antioxidant activities in B16-F10 cells. The study also explored essential oils from a broad analogs perspective. Future in-depth investigations into biomolecular or cellular antioxidant experiments will incorporate standards to further elucidate the application value of these plants.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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