Original Article

In-Vitro bioactivity testing of *Medicago sativa* L. leaf for anti-microbial, and cytotoxicity screening against Vero cells

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ABSTRACT

Anti-Microbial Drug Resistance (AMR) in pathogenic microbial organisms is a major threat to global public health. A certain study. isolated and tested a phenyl-propanol derivative from *Tabernaemontana incospicua Stapf*. (*Apocynaceae*) that not only displayed significant antimicrobial effects against the infectious *Haemophilus influenzae* 9435337A with the Minimum Inhibitory Concentration (MIC) of 62.5 µg/mL but also provides potential proof-of-ability towards combating the AMR pandemic using medicinal plants. In-vitro anti-microbial activity testing of five Alfalfa leaf extracts was evaluated using the Broth Micro-Dilution Assay and the colorimetric Microplate Alamar Blue Assay (MABA). Clinical antimicrobials Gentamicin, Vancomycin, and Fluconazole were used as controls. Cytotoxicity assays were used to determine whether cells continue to proliferate after exposure to a test compound for a specific time. The 3,4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess Vero cell viability following exposure to five Medicago sativa L. extracts. Vero cells treated with hexane, diethyl ether, and water extracts induced no decrease in the total number of cells compared to the untreated control. However, an increase of dead cells was observed after methanol, and butanol extracts treatment at 200 µg/mL. The global impact of AMR is wide and adverse, causing extended hospitalizations that amount to higher medical bills and high mortality rates.

Keywords: Anti-microbial drug resistance (AMR), Minimum inhibitory concentration (MIC), Microplate alamar blue assay (MABA), 3,4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT)

Introduction

Pharmaceutical research and development continue to struggle to meet the clinical needs of novel and effective anti-microbial agents during the ever-evolving anti-microbial drug resistance pandemic [1, 2]. The last two decades have seen the development of two novel antibiotic classes against Gram-positive bacteria: lipopeptides and oxazolidinones [3]. But the deficient existing library of newly approved anti-microbial compounds motivates an urgent need for testing natural medicines with anti-microbial activity against pathogenic and drug-resistant micro-organisms. While superbugs (Antimicrobial Drug-Resistant Pathogens) kill over 700 000 people a year [4], the insect world also possesses potential bioactivity against biofilms as in the use of the degrading enzymes derived from the rat-tailed maggot of the drone fly *Eristalis tenax* [5, 6]. The production of harmonine by the harlequin ladybird (*Harmonia axyridis*) is another example of a potential nature-derived antimicrobial agent used against malaria and tuberculosis as demonstrated by Christian Rohrich and others [7, 8].

The Gram-negative organisms are notoriously implicated in mostly fatal intra-abdominal infections, urinary tract infections, pneumonia, and bacteraemia. The strong outer membrane of Gram-negative pathogens is responsible for the globally observed antibiotic resistance [9, 10]. The innovation and development by Zurich University and Polyphor; the so-called Outer Membrane Protein Targeting Antibiotics (OMPTA), is a chimeric, cationic, and peptidomimetic antibiotics that have since demonstrated therapeutic activity against antibiotic- resistant Gram-negative pathogens [9, 11].

The Anti-microbial resistance pandemic is a global concern affecting both developed and developing countries. There is no alternative but to control the use of antimicrobial drugs, to interpret the mechanisms of resistance, and thus to engineer novel therapeutics against drug-resistant pathogens [12]. The need for effective and cost-friendly therapeutics is urgent and

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requires a multi-pronged approach that includes looking back into our past (*Sankofa*) for clues and solutions [12, 13]. The use of plants and plant-derived agents for medicinal purposes by humans and animals is old, diverse, and advanced. For humans and animals, plants play a central role in their health and wellness, vitality, and survival as plants continue to supply a large share of beneficial compounds and molecules to the food and Agri-pharmaceutical industries.

The most common bacterial Urinary Tract Infection (UTI) in women is caused by *Escherichia coli* [14, 15]. The infection can occur anywhere in the urinary tract but frequently affects the bladder, urethra, or kidneys [15]. Antibiotic drugs are invaluable in the management and treatment of infections but also in the decrease of deaths associated with infections [16, 17]. At the same time, *Enterococcus faecalis* is responsible for several nosocomial infections of the bloodstream, urinary tract, and of surgical sites [18]. Bacterial Meningitis (caused by *Streptococcus pneumoniae*) is a life-threatening condition that can lead to brain damage with sensual and cognitive impairment [19]. The species of *Candida* are notorious in the bloodstreams of hospitalized patients, as well as generally infecting our mouths, skins, and vagina [20, 21]. *S. epidermidis* is also well-known as a frequent cause of prosthetic joint infections, prosthetic valve endocarditis, and neonatal sepsis [22, 23].

Anti-biotic susceptibility testing is an effective tool in the treatment and management of infections; it allows clinicians to select the appropriate anti-microbial agent and to prescribe with precise accuracy, the bactericidal/bacteriostatic dosage [24]. The prescribed anti-biotic needs to be effective without causing significant or severe negative side effects to the person or animal or plant. The application of anti-microbial susceptibility testing for the evaluation of the potential bioactivity of natural products is one of the effective interventions instigated to curb the current surge of global AMR [25-27].

Cytotoxicity assays are tools used to determine whether cells continue to proliferate after exposure to a test compound for a specific time. Cytotoxicity is usually assessed by methods that compare either the cellular (enzymatic) function and/or cell integrity of control cells to those exposed to a test compound [28, 29]. We used a quantitative colorimetric assay based on the metabolic activity of cells to evaluate cell viability after exposure to Alfalfa extracts (MTT assay).

This paper aimed to evaluate the anti-microbial bioactivity in-vitro and to report the MIC of Alfalfa (Medicago sativa L.) leaf extracts against a panel of five clinical pathogenic strains: *E. coli, E. faecalis, S. epidermidis, S. pneumoniae, and C. albicans* using the Broth Micro-dilution method and Microplate Alamar Blue assay.

Materials and Methods

The test (plant material), the extraction, and the preparation for in-vitro bio-analysis

The plant material (dried *M. sativa* L. leaves) was purchased from a nursery and medicinal plant farm North of Pretoria (South Africa) with Batch Number MH 71(10kg) as shown in **Figure 1**. The plant material was cultivated by Zizameleni Farming based in Mamogalieskraal, Northwest Province of South Africa. The test material was cultivated using regenerative natural farming principles and the fertilizers used were all natural and certified organics. The material was air-dried and stored in a cool dry area away from light and heat. The dry, grassy, and pale green leaves were extracted using five solvents: Butanol, Diethyl-ether, Hexane, Methanol, and Water. Sixty (60) grams of powdered plant material were extracted in 1 litre (1000 mL) of every solvent. The extracts were filtered using the Buchner funnel and Whatman no.1 filter paper. The extracts were frozen at -40 °C and freeze-dried for 48 hours at a yield of 9 g of dried extracts. The dried extracts were stored at -4 °C until analysis.

Figure 1. *M. sativa* L. leaves

The in-vitro bioactivity testing of five Alfalfa leaf extracts against pathogenic micro-organisms

There are countless approaches and techniques employed for the screening of biological extracts for therapeutic potential use as antimicrobial agents. The Broth Micro-dilution susceptibility method in a 96-well microtiter plate has become the preferred method for drug susceptibility testing due to its small sample requirements and high-throughput rate [30]. Microplate Alamar Blue assay (MABA) is a colorimetric assay that can be utilized for drug-susceptibility testing based on oxidation/reduction reactions [31]. This assay uses an oxidation/reduction indicator; Alamar Blue (CellTiter-Blue®), that changes color from blue to pink, indicating bacterial growth. The color change can be read visually and quantified fluorometrically by excitation at 530 nm and detecting emission at 590 nm [31, 32].

Materials for the anti-microbial bio-activity testing of Alfalfa extracts

Enterococcus faecalis, Staphylococcus epidermidis, and *Escherichia coli* (clinical strains) were grown in Mueller-Hinton (MH) broth (Merck, USA). *Candida albicans* were maintained in Malt Extract broth (Merck, USA). *S. pneumoniae* was grown in Brain-Heart Infusion broth (Merck, USA). One microorganism colony, from an overnight streak plate, was inoculated in broth (10 mL) and allowed to grow for 16 hours (log growth phase) at 37 °C. Control drugs used: Gentamicin sulfate and Vancomycin hydrochloride (Sigma, USA) were used as positive controls against Gram-negative and Gram-positive bacteria, respectively. Fluconazole (Sigma, USA) was used as a positive control against C. albicans. Antibiotics were dissolved in ddH2O at stock concentrations of 2 mg/mL and filter sterilized (0.2 µM filter). Working concentrations were prepared in broth, depending on the antibiotics' MIC values. Test Extracts were dissolved in Dimethyl Sulfoxide (DMSO) at stock concentrations of 100 mg/mL. Working concentrations of 4 mg/mL were prepared in MH broth/Malt Extract/Brain-Heart infusion broth.

The Anti-microbial bio-activity testing procedure (broth micro-dilution assay)

50 µL of MH broth/Malt Extract/Brain-Heart infusion broth was added to all test wells (i.e., plant extracts and antibiotics),

except for the highest plant extract and antibiotic concentration wells to which 100 µL of the working concentrations were added. Serial dilutions were prepared for the plant extracts: 2 mg/mL to 125 µg/mL and for antibiotics: 64 to 0.25 µg/mL. The cultures were assessed and adjusted to a 0.5 McFarland standard (absorbance at 600 nm = 0.08-0.1; equivalent to ~1.5x108 cells/mL) and 50 µL was added to each test well.

Preparation of controls

Antibiotic/medium control (50 µL broth + 50 µL of highest antibiotic concentration). Extract color control (50 µL broth + 50 µL of highest plant extract concentration). 4% DMSO control (50 µL broth + 50 µL 8% DMSO). Microorganism control (50 µL broth + 50 µL microorganism). Plates were sealed with microplate sealing tape and incubated at 37°C for 24 h.

CellTiter-Blue® assay

After treatment, 20 µL of CellTiter-Blue® Reagent (Promega) was added to each well and incubated for 1 h. Wells were observed for the color change, where a blue color represented no growth (non-viable cells) and a pink color represented growth (viable cells). The fluorescence was read at excitation and emission wavelengths of 535 and 590 nm, respectively, using a BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA).

The in-vitro cell viability testing of five Alfalfa leaf extracts against vero cells

The 3,4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is commonly used to assess cell viability. The MTT assay is a quantitative colorimetric assay based on the metabolic activity of cells. The yellow water-soluble tetrazolium dye is reduced to a dark blue-to-purple insoluble formazan product by mitochondrial enzymes of viable cells. The insoluble formazan product is dissolved in dimethyl sulfoxide (DMSO) and can be measured spectrophotometrically at 540 nm. The amount of product produced is proportional to the number of viable cells [33-36]. The limitation of this method is that other factors can influence the metabolic activity of cells and therefore we cannot accurately measure cell death [34, 37, 38].

Measuring the integrity of cell membranes is an alternative reliable method that can be used to assess cell viability [34] Dual staining with Hoechst and Propidium iodide (PI) has been used to assess viable and nonviable cells, respectively. Hoechst 33342 is a membrane-permeant dye that binds to the minor grooves of double-stranded DNA. Hoechst 33342 is excited at a wavelength of 350 nm and emits a blue fluorescence at 461 nm. Because Hoechst is a cell-permeant dye it stains all nucleic acids and therefore gives a total cell count, i.e., live plus dead cells [39]. PI is a red fluorescent molecule that binds to DNA and is excited at 536 nm and emits light at 623 nm. PI uptake is dependent on the integrity of the cell membrane and cannot enter cells with intact membranes. The uptake and exclusion of this dye can therefore be used to discriminate between viable and nonviable cells [40-42].

Materials and methods

Vero cells were purchased from Cellonex, South Africa. DMEM low glucose cell culture medium and FBS were purchased from GE Healthcare Life Sciences (Logan, UT, USA). PBS with and without Ca2+ and Mg2+ and trypsin-EDTA was purchased from Lonza (Walkersville, MD, USA). Bis-benzamide H 33342 trihydrochloride (Hoechst) and propidium iodide (PI) was purchased from Sigma (St. Louis, MO, USA).

Image acquisition and analysis

Images were acquired using the ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices). A plate acquisition setup for a 96-well plate at a magnification of 10 times was used. Spatial distribution for the acquisition was set at 9 sites per well (3 by 3). The 9 images per site cover roughly 70% of the total well area. Filters used included DAPI (for Hoechst) and Texas Red (for PI). Analysis of acquired images was performed using the Multi-Wavelength Cell Scoring analysis module of the MetaXpress version 6.1 High-Content Image Acquisition and Analysis Software.

Cell seeding and treatment

Vero cells were seeded at a density of 5000 and 3000 cells/100 µL/well and incubated overnight at 37°C for attachment. Treatment for cytotoxicity assay was done by adding 100 µL of extract to achieve final concentrations of 50, 100, and 200 µg/mL. Cells were incubated at 37°C for 48 h. Melphalan at 6.25, 12.5, and 25 µg/mL served as the positive control as shown below in **Table 1**.

Cytotoxicity testing of Alfalfa leaf extracts procedure

After 48 h treatment medium was removed. Hoechst 33342 was diluted in 10 mL DPBS with Ca2+ and Mg2+ to a final concentration of 5 ug/mL and added to wells using 100 µL aliquots. Cells were incubated at 37°C for 30 min. PI was diluted in 1 mL PBS to a concentration of 110 µg/mL and added to wells in 10 µL aliquots before image acquisition to achieve a final concentration of 10 µg/mL.

Table 1. The Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	[50] rep1	[100] rep1	[200] rep1	[50] rep1	[100] rep1	[200] rep1	[50] rep1	[100] rep1	[200] rep1	[50] rep1	[100] rep1	[200] rep1
B	[50] rep2	[100] rep2	[200] rep2	[50] rep2	[100] rep2	[200] rep2	[50] rep2	[100] rep2	[200] rep2	[50] rep2	[100] rep2	[200] rep2
C	[50] rep3	[100] rep3	[200] rep3	[50] rep3	[100] rep3	[200] rep3	[50] rep3	[100] rep3	[200] rep3	[50] rep3	[100] rep3	[200] rep3
D	[50] rep4	[100] rep4	[200] rep4	[50] rep4	[100] rep4	[200] rep4	[50] rep4	[100] rep4	[200] rep4	[50] rep4	[100] rep4	[200] rep4
E	[50] rep1	[100] rep1	[200] rep1									
F	[50] rep2	[100] rep2	[200] rep2				POSITIVE CONTROL:					
G	[50] rep3	[100] rep3	[200] rep3	UNTREATED CONTROL			Melphalan): 6.25, 12.5, 25 µM			UNTREATED CONTROL		
H	[50] rep4	[100] rep4	[200] rep4									
A												
B	Hexane (SUNTB 1)			Diethylether (SUNTB 4)			Methanol (SUNTB 6)			Butanol (SUNTB 7)		
C	LABEL: 1			LABEL: 2			LABEL: 3			LABEL: 4		
D												
E												
F	Water (SUNTB 10)			UNTREATED CONTROL			POSITIVE CONTROL:			UNTREATED CONTROL		

G H LABEL: 5 Melphalan): 6.25, 12.5, 25 µM

Results and Discussion

Results of the screening of five extracts of Medicago sativa L. leaves for antimicrobial activity against a panel of five microorganisms (E. coli, E. faecalis, S. epidermidis, S. pneumoniae, and C. albicans), using the Broth Micro-dilution method and Microplate Alamar Blue assay

Anti-microbial drug resistance (AMR) in pathogenic bacteria is a global concern that is threatening the effectiveness of conventional clinical care systems and treatments. The World Health Organization (WHO) considers AMR "an urgent threat that requires a multisectoral action to achieve the Sustainable Development Goals (SDGs)" [43-46]. ARM also imposes a pressurizing force on major medical interventions such as surgery and cancer chemotherapy [36, 47, 48]. The poor service delivery to disadvantaged communities and villages in developing countries means a lack of water and poor sanitation which can affect infection control and prevention.

The Broth Micro-Dilution Assay was used to evaluate the anti-microbial activity of *Medicago sativa* L. leaves against five clinical strains (*E. coli, E. faecalis, S. epidermidis, S. pneumoniae, and C. albicans*). The method is favored for its highly accurate results, availability of testing plates and standard reagents, and the possibility of reading results both qualitatively (color changes) and quantitatively by Spectrophotometric techniques [24, 49, 50].

The twofold dilutions of the candidate anti-microbial agent (plant leaf extracts) were incubated in broth with the 0.5 McFarland standard adjusted organism suspension. The clinical antimicrobial agent (Vancomycin/Gentamicin/ Fluconazole). Following the 24-hour incubation period, 20 µL of CellTiter-Blue® Reagent (Promega) was added to each well and incubated for 1 h. Wells were observed for the color change, where a blue color represented no growth (non-viable cells) and a pink color represented growth (viable cells). The fluorescence was read at excitation and emission wavelengths of 535 and 590 nm, respectively, using a BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA). Fluorescence readings of 96-well plates were measured at excitation and emission wavelengths of 535 and 590 nm after the addition of CellTiter blue. Percentage inhibition for the extracts was calculated for some extracts showing inhibition of growth, but not for those that produced slight pink coloration.

All the *Medicago sativa* L. Leaves Extracts did not show inhibitory activity against *E. coli*. *Medicago sativa* L. Leaves Extracts (Hexane, Diethyl ether, Butanol) exhibited growth inhibition against *E. faecalis* with MIC (Minimum Inhibitory Concentration) of < 0.125 mg/mL. This experimental result confirms the ethnobotanical indication that *Medicago sativa* L. leaves possess anti-microbial activity against pathogenic organisms [50-53].

Medicago sativa L. Leaves Extracts (Hexane, Diethyl ether, Butanol) demonstrated inhibitory activity against *S. pneumoniae* with MIC (Minimum Inhibitory Concentration) of < 0.125 mg/mL. All the *Medicago sativa* L. Leaves Extracts did not show any inhibitory activity against *C. albicans* and *S. epidermidis*. The lack of inhibitory effect against one or more organisms is not enough to discourage further testing and deeper experimentation of *M. sativa* L.'s Anti-microbial activity against other pathogens. The experimental findings establish a scientific fact that plants can inhibit the growth and multiplication of disease-causing micro-organisms. This finding gives the religious habit of drinking herbal tea for health and wellness more value and potentiates the use of *Medicago sativa* L. as a possible alternative non-drug anti-microbial agent.

Results of cytotoxicity screening of Alfalfa extracts in vero cells

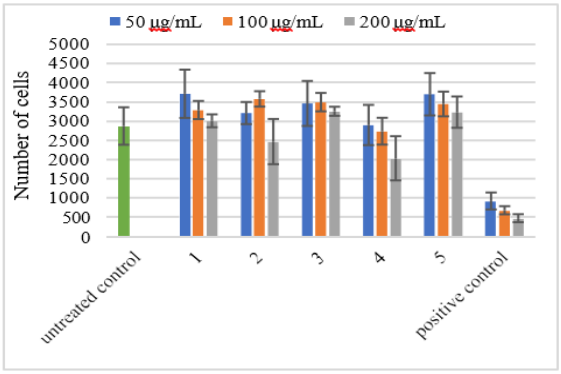

Figure 2. Total number of cells (live + dead) after treatment with extracts and positive control (melphalan).

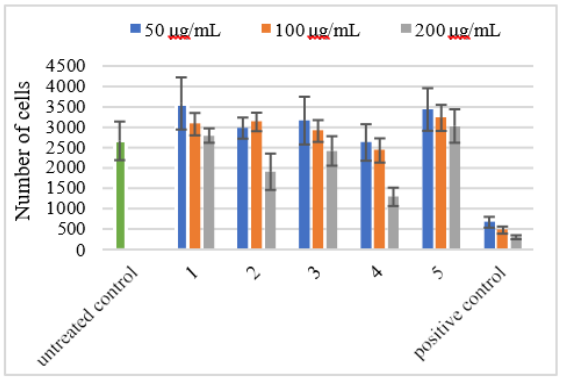

Figure 3. Number of live cells after treatment with extracts and positive control (melphalan).

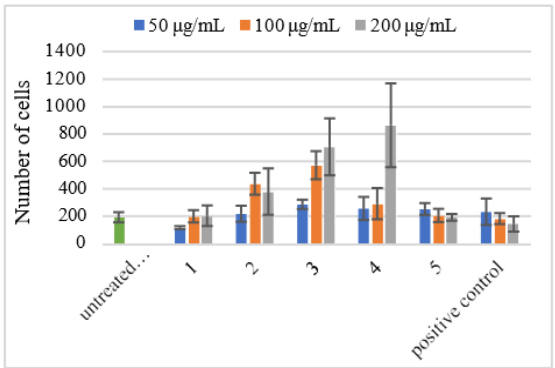

Figure 4. Number of cells with PI positive staining (dead cell number) after treatment with extracts and positive control (melphalan).

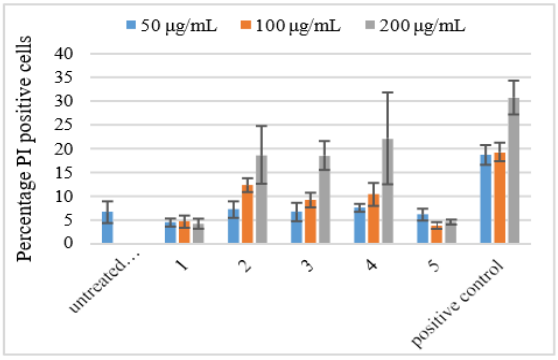

Figure 5. Percentage PI positive after treatment with extracts and positive control (melphalan).

Cytotoxicity assays are tools used to determine whether cells continue to proliferate after exposure to a test compound for a specific time. Cytotoxicity results are shown and discussed for a cell density of 5 000 cells/100 µL/well. The Total number of cells (live + Dead): Vero cells treated with hexane (1), methanol (3), and water (5) extracts did not show a decrease in the total number of cells compared to the untreated control **(Figure 2)**. A decrease in the total number of cells was observed for diethyl ether (2) and butanol (4) extracts at 200 µg/mL **(Figure 2)**. The Number of Live cells: Vero cells treated with hexane (1) and water (5) extracts did not show a decrease in the number of live cells compared to the untreated control **(Figure 3)**. A decrease in the number of live cells was observed for diethyl ether (2), methanol (3), and butanol (4) at a concentration of 200 µg/mL compared to the untreated control **(Figure 3)**. The Number of PI-positive Cells (dead cells): Vero cells treated with hexane (1) and water (5) did not show an increase in the number of PI-positive cells **(Figure 4)**. Diethyl ether (2), methanol (3), and butanol (4) showed an increase in the number of PI-positive cells **(Figure 4)**. The Percentage PI positive: Vero cells treated with hexane (1) and water (5) did not show an increase in the percentage of PI-positive cells (6. Diethyl ether (2), methanol (3), and butanol (4) showed a dose-dependent increase in the percentage of PI-positive cells **(Figure 5)**. We report therefore report that the hexane and water extracts demonstrated no evidence of toxicity or cytostatic activity. While the diethyl ether, methanol, and butanol extracts demonstrated toxicity at 200 µg/ml but not lower concentrations.

Plants and other natural sources continue to provide a diverse variety of compounds, analogues, and derivatives that can be used to formulate and develop the much-needed anti-microbial drugs that can combat antibiotic-resistant pathogens [54]. Anti-microbial susceptibility testing of medicinal plants is not only used to confirm the ethnobotanical literature but also to evaluate the plant as a prospective anti-microbial agent against drug-resistant clinical pathogens. The use of *alfac-facah* for medicinal purposes includes use as a neuroprotective, anti-microbial, anti-cancer, and cholesterol-lowering agent [50, 51, 55, 56]. Newly isolated triterpenoid saponins from *M. sativa* L. demonstrated neuroprotective potential against hydrogen peroxide-induced SH-SY5Y (Human Neuroblastoma) cell death compared to the hydrogen peroxide-treated group [51]. The toluene and methyl tert-butyl ether (MTBE) extracts of *M. sativa* L. demonstrated *in-vitro* cytotoxic effects on two murine leukemia cell lines P388, whence the extracts also induced programmed cell death by activation of the caspase-3, leading to poly ADP ribose polymerase cleavage (PARP) [54].

Conclusion

Despite the good recording of negative clinical-drug-to-herbal-drug interactions, medicinal plants continue to play a critical role in disease prevention and drug development [57]. The regular and consistent consumption of herbal infusions was a key action that our ancestors used to flavor water and also to siphon nutrients and secondary metabolites (phytochemicals) from plants and herbs availed by nature. The native approach to processing roots and tree barks included preparation of decoctions, some fomentations for the skin, and soaking or irrigating a specific body area. The primitive hunter was also proficient in dealing with poisonous/venomous snake and insect bites, thereby using poultice or cataplasm of macerated plant material, and wrapping it against the skin or an area of much swelling probably using a leaf or flexible bark as a bandage.

A large library of the medicinal plants used for medicinal purposes still resides mostly within our traditional healers, herbalists, and medicine men/women (*li-Ngaka, Lingaka-Tjhitja, Pepeduma, Makherenkhoa, Makhehla, le Baloi)*. The conventional medical care system still needs to integrate traditional or native medicinal approaches into its standard operating procedures. Advanced native Health care systems such as Ayurveda, homeopathy, Yunani Medicine, Phytotherapy, and Herbal Medicine are still largely isolated and not included in the scope of conventional medical care.

We rely on the results of our experiments reported here, to suggest that the perpetual drinking of a herbal beverage (tea) made from *Medicago Sativa/Alfalfa* leaf infusion for health and wellness purposes is hereby scientifically confirmed. Although we understand that laboratory-based conditions are far from a true reflection of the conditions *in-vivo,* we are confident to recommend the further testing of *Medicago sativa*-derived phytochemicals, as our experiments and experiments of others, demonstrate a significant therapeutic potential induced by the reliable agricultural fodder: *Medica Sativa/Lucerne/Alfac-facah*.

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Ethics statement: None

References

1. Bledzhyants GA, Mishvelov AE, Nuzhnaya KV, Anfinogenova OI, Isakova JA, Melkonyan RS, et al. The effectiveness of the medical decision-making support system" electronic clinical pharmacologist" in the management of patients therapeutic profile. Pharmacophore. 2019;10(2):76-81.

2. Majumder KK, Sharma JB, Kumar M, Bhatt S, Saini V. Development and validation of UV-Visible spectrophotometric method for the estimation of curcumin in bulk and pharmaceutical formulation. Pharmacophore. 2020;10(1):115-21.
3. Pangerciḉ A, Bukovski-Simonoski S, Barsiḉ B. Lipopeptides and oxazolidinones--novel antibiotics in MRSA infection treatment. Lijec Vjesn. 2010;132:11-3. Available from: https://europepmc.org/article/med/20715711.
4. World Health Organization. The global burden of disease: 2004 update. Available from: https://www.who.int/healthinfo/global_burden_disease/GBD_report_2004update_full.pdf?ua=1
5. Hirsch R, Wiesner J, Bauer A, Marker A, Vogel H, Hammann PE, et al. Antimicrobial peptides from rat-tailed maggots of the drone fly eristalis tenax show potent activity against multidrug-resistant gram-negative bacteria. Microorganisms. 2020;8(5):626.
6. Aldhairyan AH, Alyami SSH, Alsaad AMS, Al Shuqayfah NI, Alotaibi NA, Mujammami NM, et al. Gastroesophageal reflux disease: Diagnosis and management approach, literature review. World J Environ Biosci. 2022;11(1):1-3. doi:10.51847/EvuxMWxAai
7. Almuhanna MA, Alanazi MH, Ghamdi RNA, Alwayli NS, Alghamdi ISG, Qari AA, et al. Tachycardia evaluation and its management approach, literature review. World J Environ Biosci. 2022;11(1):4-8. doi:10.51847/7maH6sWjQy
8. Röhrich CR, Ngwa CJ, Wiesner J, Schmidtberg H, Degenkolb T, Kollewe C, et al. Harmonine, a defence compound from the harlequin ladybird, inhibits mycobacterial growth and demonstrates multi-stage antimalarial activity. Biol Lett. 2012;8(2):308-11.
9. Luther A, Urfer M, Zahn M, Müller M, Wang SY, Mondal M, et al. Chimeric peptidomimetic antibiotics against Gram-negative bacteria. Nature. 2019;576(7787):452-8. doi:10.1038/s41586-019-1665-6
10. Almohmmadi GT, Bamagos MJ, Al-Rashdi YJR, Alotaibi NS, Alkiyadi AA, Alzahrani AM, et al. Literature review on polycythemia vera diagnostic and management approach. World J Environ Biosci. 2022;11(1):9-12. doi:10.51847/ipOt4R1qlz
11. Alqurashi AMA, Jawmin SAH, Althobaiti TAA, Aladwani MNMFA, Almuebid AME, Alharbi JFA, et al. An overview on nasal polyps' diagnosis and management approach. World J Environ Biosci. 2022;11(1):13-6. doi:10.51847/gde2ofOvaO
12. Ashraf K, Halim H, Lim SM, Ramasamy K, Sultan S. In vitro antioxidant, antimicrobial and antiproliferative studies of four different extracts of Orthosiphon stamineus, Gynura procumbens and Ficus deltoidea. Saudi J Biol Sci. 2020;27(1):417-32.
13. Alhazmi RA, Khayat SK, Albakri MH, Alruwaili WS, Bayazed HA, Almubarak SA, et al. An overview on the assessment and management of polycystic ovarian syndrome. World J Environ Biosci. 2022;11(1):17-23. doi:10.51847/Yaaa2745ZY
14. Alsayed MA, Alhassan OMA, Alzahrany AM, Mutanbak HIM, Alamoudi AA, Eid SM, et al. An overview on lumbar disc herniation on surgical management approach. World J Environ Biosci. 2022;11(1):24-9. doi:10.51847/OJ2dQINEwx
15. Das S. Natural therapeutics for urinary tract infections—A review. Future J Pharm Sci. 2020;6:1-3.
16. World Health Organization. 2020. Antimicrobial resistance 13.10.2020. Available from: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance
17. Alshehri FS, Alotaibi FF, Alghanim NS, Almutairi FT, Alsuwailem HS, Darwish EG, et al. Status epilepticus diagnostic and management approach in emergency department. World J Environ Biosci. 2022;11(1):30-3. doi:10.51847/OsSd2wuQQY
18. Zaheer R, Cook SR, Barbieri R, Goji N, Cameron A, Petkau A, et al. Surveillance of enterococcus spp. reveals distinct species and antimicrobial resistance diversity across a One- Health continuum. Sci Rep. 2020;10(1):3937.
19. Zhao S, Zhang Z, Xu D, Wang Y, Li L. Selective loss of brain-derived neurotrophic factor exacerbates brain injury by enhancing neuroinflammation in experimental streptococcus pneumoniae meningitis. Front Immunol. 2020;11:1357.
20. Erum R, Samad F, Khan A, Kazmi SU. A comparative study on production of extracellular hydrolytic enzymes of Candida species isolated from patients with surgical site infection and from healthy individuals and their co-relation with antifungal drug resistance. BMC Microbiol. 2020;20(1):1-2. doi:10.1186%2Fs12866-020-02045-6
21. Dirican S. A look at the change in water occupancy rates of gölova dam lake, Turkey. World J Environ Biosci. 2022;11(1):34-6. doi:10.51847/3u8KMQDDzQ
22. Hellmark B, Söderquist B, Unemo M, Nilsdotter-Augustinsson Å. Comparison of staphylococcus epidermidis isolated from prosthetic joint infections and commensal isolates in regard to antibiotic susceptibility, agr type, biofilm production, and epidemiology. Int J Med Microbiol. 2013;303(1):32-9. doi:10.1016/j.ijmm.2012.11.001
23. Shaheen RS, Alsaffan AD, Al-Dusari RS, Helmi RN, Baseer MA. Self-perceived oral hygiene and periodontal health among dental and medical students, dentists and physicians in KSA. Ann Dent Spec. 2022;10(1):126-32. doi:10.51847/NVcZEJ0YBV
24. Barónková S, Apers S, Berghe DV, Cos P, Vermeulen P, Van Daele A, et al. An ex-vivo angiogenesis assay as a screening method for natural compounds and herbal drug preparations. Planta Med. 2004;70(10):887-92.
25. Schumacher A, Vranken T, Malhotra A, Arts JJ, Habibovic P. In vitro antimicrobial susceptibility testing methods: Agar dilution to 3D tissue-engineered models. Eur J Clin Microbiol Infect Dis. 2018;37:187-208.
26. Vamvuka D, Teftiki A, Sfakiotakis S. Investigating the valorisation of refused derived fuel for energetic uses through its co-gasification with woody wastes. World J Environ Biosci. 2022;11(1):37-44. doi:10.51847/2fiOEjSU7L
27. Alsharif SB, Bahanan L, Almutairi M, Alshammry S, Khalifa H. Retrospective assessment of dental implant-related anatomical structure perforations using cone beam computed tomography. Ann Dent Spec. 2023;11(1):21-30. doi:10.51847/jEyOudiF94
28. Cummings BS, Schnellmann RG. Measurement of cell death in mammalian cells. Curr Protoc Pharmacol. 2004;25(1):12-8. doi:10.1002/0471141755.ph1208s25
29. Chandra S, Meel RK. A systematic comparative study of morinda tinctoria and vitex negundo for their anti-ulcerogenic potential. World J Environ Biosci. 2022;11(1):45-52. doi:10.51847/aNF9QSYDRo

30. Pauli GF, Case RJ, Inui T, Wang Y, Cho S, Fischer NH, et al. New perspectives on natural products in TB drug research. Life Sci. 2005;78(5):485-94.
31. Collins LF, Franzblau SG. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium. Antimicrob Agents Chemother. 1997;41(5):1004-9.
32. Poornachitra P, Maheswari U. Analysis of clinical symptoms in patients with oral submucous fibrosis. Ann Dent Spec. 2023;11(1):1-6. doi:10.51847/iRUEgUex6m
33. Verma P, Pandian SM. Prevalence of endodontically treated posteriors in patients undergoing orthodontic treatment-cross-sectional radiographic evaluation. Ann Dent Spec. 2022;10(1):1-6. doi:10.51847/VtxY3JqaJ5
34. Carolina LE, Varela-Ramirez A, Aguilera RJ. Differential nuclear staining assay for high-throughput screening to identify cytotoxic compounds. Curr Cell Biochem. 2011;1(1):1-14.
35. Vega-Avila E, Pugsley MK. An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. InProc West Pharmacol Soc 2011 Jan 1 (Vol. 54, No. 10, p. 4).
36. Zahid TM, Khan NS. Myrrh and chlorhexidine mouthwashes comparison for plaque, gingivitis and inflammation reduction: A 3-arm randomized controlled trial. Ann Dent Spec. 2022;10(1):39-46. doi:10.51847/ajwgutvUNV
37. Mady M, AlArabi AA, Turkistani AM, AlSani AA, Murad GS, AlYami AS, et al. The role of laser in modern dentistry: Literature review. Ann Dent Spec. 2022;10(1):133-6. doi:10.51847/GHfzVsoiAa
38. Maneea ASB, Alqahtani AD, Alhazzaa AK, Albalawi AO, Alotaibi AK, Alanazi TF. Microbiological effect of various concentrations of sodium hypochlorite (NaOCL) during endodontic treatment: A systematic review. Ann Dent Spec. 2023;11(1):95-101. doi:10.51847/7CZTguksH9
39. Chazotte B. Mounting live cells onto microscope slides. Cold Spring Harb Protoc. 2011;2011(1):pdb-rot5554. doi:10.1101/pdb.prot5554
40. Ciancio G, Pollack AL, Taupier MA, Block NL, Irvin 3rd GL. Measurement of cell-cycle phase-specific cell death using Hoechst 33342 and propidium iodide: Preservation by ethanol fixation. J Histochem Cytochem. 1988;36(9):1147-52.
41. Crowley LC, Scott AP, Marfell BJ, Boughaba JA, Chojnowski G, Waterhouse NJ. Measuring cell death by propidium iodide uptake and flow cytometry. Cold Spring Harb Protoc. 2016;2016(7):pdb-rot087163. doi:0.1101/pdb.prot087163
42. Pimple NS. Virtual population analysis and recruitment pattern of *Osteobrama vigorsii* (Sykes, 1839) from nira river, bhor maharashtra. World J Environ Biosci. 2022;11(1):53-9. doi:10.51847/QslcwlezoR
43. Mulu E, Jenber AJ, Tesfaye A, Belay B. Integrated management of onion thrips on onion, mecha district, Ethiopia. World J Environ Biosci. 2023;12(1):32-40. doi:10.51847/bbmj8P5dll
44. World Health Organization. Factsheet on SDG's: Antimicrobial resistance. 2017, Viewed 16.11.2020. Available from: https://www.euro.who.int/en/health-topics/health-policy/sustainable-development-goals/publications/2017/fact-sheets-on-the-sustainable-development-goals-sdgs-health-targets/fact-sheet-on-the-sdgs-antimicrobial-resistance-2017.
45. Shetty B, Chauhan RS, Vishwas P, Rathi N, Krishnapriya N, Tirupathi S. Antimicrobial efficacy of curcumin modified zinc oxide eugenol against endodontic pathogens. Ann Dent Spec. 2022;10(1):47-51. doi:10.51847/1DcKJ12DSy
46. Abdulrahman BI, Alanazi AJ, Alanazi AJ, Idrees FF, Abuabah A, El Mansy IT, et al. Natural therapeutic agents in the treatment of recurrent aphthous ulcer: A systematic review and meta-analysis. Ann Dent Spec. 2022;10(1):78-86. doi:10.51847/dXjgbEIZDw
47. Zigmantavičius J, Kilinskaitė G, Leketas M. Dimensional changes of buccal bone after immediate implantation using different grafting materials: A systematic review. Ann Dent Spec. 2023;11(1):7-15. doi:10.51847/vykVdVPn8e
48. WHO. Antimicrobial resistance. 2020 October 13. Available from: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance.
49. Aodh AM, Al-Marshedi AA. Williams-beuren's syndrome: A case report in Prince Sultan Military City, Riyadh, Saudi Arabia 2022. World J Environ Biosci. 2023;12(1):20-3. doi:10.51847/dQxngBeVLQ
50. Govindaraj A, Paulpandian SS, Shanmugam R. Effect of chlorhexidine and fluoride varnish on the incidence of white spot lesion in orthodontic patients. Ann Dent Spec. 2023;11(1):35-9. doi:10.51847/RgSlwwnDkR
51. Liu XG, Sun YQ, Bian J, Han T, Yue DD, Li DQ, et al. Neuroprotective effects of triterpenoid saponins from Medicago sativa L. against H2O2-induced oxidative stress in SH-SY5Y cells. Bioorg Chem. 2019;83:468-76.
52. Gowda B, Gurusiddappa LH, Kalikeri S. Study on occupational health hazards of municipal solid waste workers - A review. World J Environ Biosci. 2023;12(1):24-31. doi:10.51847/dmEF1XWBtq
53. Chidambaranathan AS, Culathur T. A prospective clinical study to evaluate the effectiveness of acupuncture treatment for temporomandibular joint muscular disorder. Ann Dent Spec. 2022;10(1):34-8. doi:10.51847/172V2CMsF3
54. Rafińska K, Pomastowski P, Wrona O, Górecki R, Buszewski B. Medicago sativa as a source of secondary metabolites for agriculture and pharmaceutical industry. Phytochem Lett. 2017;20:520-39.
55. Al Hamazani A, Al Robayaan A, Al Fuhaid A, Al Mutairi F, Mwais M, Khahtani NSA, et al. Dental home care needs among homebound individuals at Prince Sultan Military Medical City. Ann Dent Spec. 2023;11(1):78-82. doi:10.51847/1SbFcXeuVl
56. Sabbahi DA. Systematic review of different outcomes for dental treatment provided to children under general anesthesia. Ann Dent Spec. 2022;10(1):13-33. doi:10.51847/XjoKWslc7T
57. Siddiqui AJ, Danciu C, Ashraf SA, Moin A, Singh R, Alreshidi M, et al. Plants-derived biomolecules as potent antiviral phytomedicines: New insights on ethnobotanical evidences against coronaviruses. Plants. 2020;9(9):1244. doi:10.3390%2Fplants9091244