Standardization of in-vitro methods for determination of antimicrobial properties of essential oils

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Essential oils (EOs) have been recognized as well tolerated substances with great commercial value that finds applications in traditional medicine. This study systematically reviews various methods published from 2016 to 2020 that were employed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils. The main question was, 'What are the most reliable in-vitro methods to determine MIC and MBC of EOs'? The reported outputs of the search terms for the colony-forming unit (CFU), MIC, MBC, and the zone of inhibition (ZOI) were used to assess the methods.121 studies reported 'Antibacterial', 90 'Antifungal', 110 'Antimicrobial', 71 'ZOI', 17 'CFU', and 35 'MIC and MBC'. Forty-seven studies were selected according to the criteria for analysis in this review. This review indicated that each study requires its appropriate method. The antimicrobial efficacy of herbal extracts or EOs was not comparable to those of the antibiotics. The use of standard microbial strains is essential when clinical strains are included in the research. Based on the contents and analysis of the published articles, it is clear that designing antimicrobial experiments using EOs would produce more reliable results than those of the extracts. Based on the literature review and our experience, we suggest parameters to consider while determining antimicrobial properties of essential oils.

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Introduction

Antibiotics are of prime importance in controlling bacterial infections, however, the emergence of bacterial resistance has threatened their efficacy [1]. Fifteen categories of antibiotics are known to interfere with the essential physiological or metabolic functions of microorganisms. The resistance phenomenon is unfortunately persistent [2]. A growing number of pathogens characterized with antibiotic resistance poses a considerable threat to humans. The global response must pay attention to investigate novel approaches to handle the crisis of antimicrobial resistance [3]. Treatment of bacterial infections is hindered by the development of antimicrobial resistance leading to render the treatment measures less effective [4]. There is a remarkable increased number of multiple drug resistant (MDR) pathogenic bacteria, as methicillin-resistant Staphylococcus *aureus* (MRSA) and clinically isolated strains of *Escherichia coli* characterized with drug resistance [5]. The use of antibiotics in human healthcare or animal feed has greatly improved human living standards and the quality of animal products. It has also been employed in the categorization of bacterial-resistant strains into a wide variety of antibiotic groups. The increasing microbial resistance to various drugs has prompted a requirement

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for new antimicrobial agents. Antimicrobial resistance hampers the effective treatment of a range of bacterial, viral, and fungal infections. Bacterial resistance poses a considerable economic impact. Various synthetic compounds pose undesirable adverse effects, and hence new molecules of the least side effects are the ultimate goal of the researchers. New effective agents are needed against common pathogens that are resistant to conventional antimicrobial agents. Newly developed effective antimicrobial agents remain a challenge because microbial infection is a persisting human health threat. The resistance of various bacterial species to antibiotics is launching new windows for alternative treatment strategies. In these windows, the essential oils (EOs) from medicinal plants are attracting attention. Safe and effective antimicrobial agents acting against a wide variety of bacterial infections by way of therapeutical or prophylactical treatment is still of top priority in biomedicine. The clinical outbreak of yeast infections has increased in recent years. EOs from medicinal plants as antimicrobial compounds have great potential against pathogens. *Candida* species, the causative agents of most nosocomial infections can be controlled by EOs [6]. Notable drug resistance is increasingly seen among microbial species as a result of extensive use of broad-spectrum antimicrobial agents. Synergy might be an efficient strategy in clinical treatments against MDR bacteria. Medicinal plants have been relied upon by folk medicine for centuries. Valuable chemotherapeutic agents used to treat various diseases or pathogenic infections have been extensively prepared from medicinal herbs markedly because their effectiveness, safety, and straightforward availability at affordable cost, particularly at the living standard levels of developing countries [7]. The utilization of herbs to control or treat a variety of diseases in rural areas is documented in most countries. The tolerance of patients to most herbal medicines with fewer unintended consequences than synthetic medicine is well known. The side effects of herbal products are typically fewer than synthetic drugs, and hence could be reliably used over time [8]. For their rich secondary metabolites like flavonoids, alkaloids, tannins, saponin, and phenolic compounds, the new herbal antibacterial compounds became significant treatment alternatives against pathogens. The food and pharmaceutical industries have widely investigated the characteristics of bioactive compounds from EOs and herbal extracts. EOs have greatly attracted a wide range of activities in biological and chemical fields to further explore their potentials as natural alternatives to chemical food preservatives. Antibacterial packaging has gained the advantage of the applications of the volatile state of EOs in the systems. The wider the knowledge of EOs compositions and food packaging, the greater will be the efficacy of EOs in food preservation. This will target their application in food industries. The use of plants or plant products by humans for medicinal purposes dates long back in history. The majority of the nations in the world consume herbal medicines in different preparations for their health and safety requirements [9]. The synergism between the secondary metabolites of plants and antibiotics is now developed in addition to new concepts like efflux pump inhibitors [10]. Natural products, as the latest important therapeutics and future medicines, is highlighted by the world's annual use of herbal medicines [11]. High volatility and complex composition are characteristics of EOs or aromatic compounds derived from vegetable materials. EOs possess antimicrobial actions to be extensively used in food, agricultural, pharmaceutical, and cosmetic products. EOs and chemical or aromatic compounds isolated from them, like terpenes and terpenoids, have shown remarkable antimicrobial characteristics against a range of pathogens [12]. Cell damage brought about by EOs is attributed to their chemical composition and cytotoxic effects. The cell and cytoplasmic membranes are affected by lipophilic effect of EOs. Many researchers have extensively documented antimicrobial effects of EOs and their constituents. Despite the reports available on the mechanism of action of a couple of volatile oil constituents, reliable determination methods of their antimicrobial properties remain obscure. The membrane becomes permeable by disruption of the structure of the carboxylic acid, polysaccharide, and phospholipid layers EOs [13]. The EOs not only target the pathogens specifically but also affect eukaryotic cells reversibly or irreversibly. Apoptosis, organ failure, and necrosis can be brought about by the cytotoxic effect of Eos [14]. Therefore, care should be taken on the daily use of EOs, within the prescribed intake limits specified by the authorities. This data is of especial importance when the effect of EOs is determined against various microorganisms. The combined or synergic action of EOs with other antimicrobial compounds may be studied. Their interaction may also be studied with food matrix components. The lack of scientific knowledge on the normal medicinal use of herbal products regarding the cellular and molecular factors affecting biological activities prompted us to design and introduce appropriate techniques to determine the antimicrobial activities of EOs. This review is aimed at giving a summary of current knowledge about the methods determining the antibacterial activities of EOs.

Methods

Definitions

A search in the literature published from 2016 to 2020 in the English language was carried out on ScienceDirect, PubMed, and Medline databases. The search was carried out for in-vitro studies, and the reported outputs of CFU, MIC, MBC, and ZOI were used to assess the methods. A few methods are available for antibacterial activity assessment of EOs. The Agar disc diffusion method is

one of the well known methods employed for routine antimicrobial susceptibility tests where the diameters of the zone of growth clearance or growth inhibition zone are measured. Most researchers employed disc diffusion and broth dilution methods, while some others used rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay or resazurin microtiter plate assay (REMA) method for MIC determination. The minimum bactericidal concentration was defined as MBC [15]. The level of a compound concentration leading to no sign of visible microbial growth compared to the control upon subculturing was defined as the MBC for bacteria, or minimum fungicidal concentration (MFC) for fungi [16]. The lowest concentration inhibiting growth and no visually detectable color change in the colorimetric test was termed MIC. The bacteriostatic effect was referred to as the observation of some microbial colonies on the agar plate, while the bactericidal effect of the extracts was attributed to the concentration at which absence of microbial growth was noted [17]. MIC was the lowest concentration of sample inhibiting complete bacterial growth. The minimum microbicidal concentration (MMC) was the highest sample dilution yielding no post sub-culturing growth [18]. The lowest concentration of drug leading to no visible growth was defined as MIC. The MBC was followed subsequent to determination of the MIC. The concentration causing 99.9% death of the initial inoculum was defined as MBC [19]. Inhibition of bacterial growth by the lowest hop concentration was defined as MIC [20]. We have employed various methods to define and determine the MIC and MBC of various essential oils accordingly [21–29].

Literature sources

At least 100 articles from reliable databases such as ISI, PubMed, and Scopus have been collected over the past 4 years. All articles were read considering the following parameters:

- Does the plant have a voucher no.?
- Date and place of collection.
- Which organs of the plant are studied and at what growth stage?
- The effects of different drying methods such as temperature, shadow, sun, or . . ..
- Is the type of extraction solvent correct, and has it been considered in the next steps?
- Was the sample of the stock solution made with appropriate concentrations?
- Was the storage solution made by weight or volume?
- What methods were used to investigate the antimicrobial effect?
- Is the accuracy or validity of the selected method correct?
- What are the probable problems associated with the presently used methods?
- Was the chemical composition of the test material known?
- Was a standard chemical compound or antibiotic or a microbial strain used as a control?
- Was the statistical design of the experiment appropriate?
- Was the toxicity of the test material determined?

3- Summarizing and determining the exact problems according to the discrepancies with the instructions.

4- Suggestions for solving the problem and corrections are made in the form of an Expert panel.

Results

Interference of solvents and diluents

Nutmeg, the seed kernel inside *Myristica fragrans* Houtt. (Myristicaceae) the fruit is known to possess various pharmacological activities. In Cameroon, it is used as a cake flavor. Antibacterial and antibiotic activities of methanolic extract (ME) of the crude seed kernel from *M. fragrans* Houtt., and its five fractions (a–e) and 3',4',7-trihydroxyflavone were tested against an array of Gram-negative multidrug resistant (MDR) bacteria. MIC and MBC were determined on a selected panel of bacteria using modified rapid INT colorimetric assay. The same assay was also conducted with antibiotics combined with the extract, and with or without isolated chemical components. Two-fold serial dilutions of the extract were prepared to obtain a concentration range of 1.22–625 μg/ml. 100 μl from each concentration of the extract was added to each well in a 96-well plate containing 95 μl of Mueller Hinton broth (MHB) and 5 μl inoculum of 1.5×10^6 CFU/ml. The preliminary analyses showed no growth inhibition of the test organism at 1% (v/v) dimethyl sulfoxide (DMSO) and hence, per well DMSO concentration of >1% was maintained. The negative control contained MHB (195 μl) and standard inoculum (5 μl). The plates, in triplicate, were aseptically sealed and agitated on a plate shaker to mix the contents. Each sample was added with 40 μl of 200 μg/ml *p*-iodonitrotetrazolium chloride and incubated for 30 min at 37°C to determine the MIC. The presence of viable bacteria brought about reduction of yellow dye to pink color. MIC was the lowest concentration of the sample preventing the color change and exhibiting complete bacterial growth inhibition. Minimum microbicidal concentration (MMC) was determined by sampling 5 μl of liquid from each well showing no change in color, and plating on Mueller Hinton (MH) agar and incubating for 48 h at 30 °C or 24 h 37 °C for *Microsporum audouinii* or other organisms respectively. The lowest sample concentration was defined as MMC that inhibited microbial growth on sub-culturing. Most of the Gram-negative bacterial strains did not grow at an MIC range of 32–1024 μg/ml. The lowest MIC value of 4 μg/ml and the highest MBC value of 16 μg/ml were recorded against *Providencia stuartii* ATCC 299645 with 3′,4′,7-trihydroxyflavone respectively [18]. DMSO-

MHB at a 10:90 ratio was used to dissolve the plant extract and gentamicin. DMSO below 2.5% concentration did not affect microbial growth. 100 µl from the plant extracts dilutions containing 80–1.25 mg/ml concentration range were added to the wells in a sterile 96-well plate. Each well was then inoculated with 100 µl of 1.5×10^6 CFU/ml inoculum prepared in MHB. DMSO-MHB and bacterial inoculum served as negative growth control. The positive reference antibiotic control consisted of MHB containing 80–1.25 µg/ml of gentamicin. MHB for medium sterility and the bacterial inoculum were additional controls. The plate sealed with sterile plate sealer were mixed by agitation on a shaker. 40 µl of 200 µg/ml *p*-iodonitrotetrazolium chloride were mixed with the samples and processed for a 30-min incubation. The live bacteria converted the dye from a yellow color to pink. Some obstacles were interfering with the correct reading of microplates with a microplate reader. These obstacles included precipitation of extracts compounds, microbial cells clumping, and the green color of the extract. Therefore, MIC and MBC determination was made by visible examination of the color change of the INT chloride. The well with no change in color was taken as MIC. To determine MBC, 50 µl aliquots from the lowest concentration exhibiting growth inhibition, was added to 150 µl of the broth and incubated at 37 °C for 48 h. MBC was determined by incubating 150 µl of the broth with 50 µl aliquots from the lowest MIC concentration upwards at 37 °C for 48 h. The lowest extract concentration inhibiting color change upon the addition of INT was taken as MBC [30]. The MIC and MBC of the ethanolic extract of *Curcuma xanthorrhiza* were determined against *Mycobacterium tuberculosis* H37Rv, using microdilution and disc diffusion methods respectively. MIC was determined with Resazurin microtiter plate assay (REMA) in 7H9-S medium containing Middlebrook broth, glycerol (0.5%), and Casitone (0.1%), supplemented with oleic acid albumin dextrose catalase. 100 µl of Middlebrook 7H9 broth was added into each well in a 96-well ELISA plate. Two-fold serial dilutions of powder-free *C. xanthorrhiz*a ethanol extract were prepared in Middlebrook 7H9 broth. Serial two-fold drug dilutions were prepared from 3200 µg/ml. Rifampicin was used as a standard at 10 µg/ml concentration. 100 µl bacterial suspension at 6×10^6 CFU/ml of *M. tuberculosis* H37Rv were added to each well, and the plate was wrapped with aluminum foil. The plate was incubated for 7 days at 37 °C followed by the addition of 30 µl of resazurin solution at 100 µg/ml concentration. The plate was further incubated overnight while wrapped in aluminum foil. A color change from blue to pink or colorless was regarded as an indication of the presence and growth of viable bacteria. The upper limit for the MIC was the lowest extract concentration that prevented color change. The highest concentration of the ethanolic extract of *C. xanthorrhiza* exhibiting a change of blue to pink color was taken as the lower limit. The paper disc diffusion method was carried out by slowly absorbing the same serial dilutions in a distilled water solution, into the sterilized 8 mm paper discs. A Petri plate was streaked with 10^6 CFU/ml concentration of *M. tuberculosis* strains H37Rv in Middlebrook 7H9 broth. The discs were placed on the surface of the agar in petriplate and incubated for 24 h at 37 °C. Sterile distilled water served as the control. MBC was defined as the lowest concentration of the extract causing a halo around the discs [15].

Synergic interactions

The synergic interactions between antibiotics and phenolic-rich compounds of grape pomace extract were evaluated against strains of *S. aureus* and *E. coli* isolates using the checkerboard method. To find out the chemical components of the extract imparting the synergic effect, RP-HPLC was used to identify phenolic compounds and their relative abundance. Extract combined with the identified compounds in pure forms were also evaluated. MIC against all used strains of four different classes of antibiotics was determined. Ampicillin and oxacillin of the β-lactam class; norfloxacin, ciprofloxacin, and levofloxacin as representatives of the fluoroquinolones class; nalidixic acid for quinolone; tetracycline as a representative of tetracycline family; and chloramphenicol representing amphenicol class. The MIC of grape pomace extract was determined. The phenolic compounds of pomace identified as syringic acid were vanillic acid, gallic acid, luteolin, kaempferol, quercetin, (+)-catechin, and (−)-epicatechin. The MIC was determined by the microdilution method following the Clinical and Laboratory Standards Institute (CLSI) assay procedure. DMSO was used to dilute pomace extract to 300–3000 µg/ml. 0.75–3000 µg/ml of antibiotic or 200–10 000 µg/ml of pure phenolic compound diluted in DMSO, was added to MH broth (188 µl) in the microplate well followed by the addition of 2 µl of bacterial suspension of 1×10^8 CFU/ml concentration. Solvent and sterility controls were also used in the wells. The OD_{600} nm was measured after 24 h of incubation at 37 °C. The checkerboard method was used to test the synergism. Compounds most likely responsible for synergism could be identified by analyzing the results of the mixture between the whole extract and pure compounds. 25 µl from each of the two-fold serial dilutions of the extract was added in each vertical row in a 96-well microplate. Each horizontal row was seeded with the same quantity of antibiotic or pure compound dilutions. One agent was allotted to the first two horizontal and vertical rows. The following rows were dedicated to a known quantity of an agent and ascending concentrations of the other. MICs for each agent and the bacteria were the basis of the selection of the concentration range. A 47–3000 µg/ml range of the pomace extract concentrations and a 3–3000 µg/ml range of the antibiotics were used. A hundred microliters of MH broth and 10 µl of 10^8 CFU/ml of the bacterial suspension were mixed in each well and maintained at 37 °C for 24 h

followed by measurement of $OD_{600\ nm}$. The plates were made in triplicates. The FICs for the extract or antibiotic were determined by dividing (MIC extract + antibiotic) by $MIC_{extract}$ or $MIC_{antibiotic}$, respectively. For each combination, the sum of both FIC values was used to calculate the FIC index. The synergic, additive and antagonistic effects were interpreted as FIC index of ≤ 0.5, $0.5-\leq 4$, and >4, respectively. The same formula was applied to calculate the combinations of the extract with each phenolic compound. The relative abundance of phenolic compounds was not found to be correlated to the obtained synergic effect. This implies the existence of a multiobjective action in the synergic effect. Synergistic effect concentrations of the grape pomace extract and antibiotics combination were not toxic to the HeLa cell line. The results suggest a tool for testing these combinations in animal models to boost the efficacy of various classes of antibiotics [31].

Biofilm inhibition by essential oil

Effective antibacterial activity was reported with fatty acids extracts of *Lucilia sericata* larvae against *S. aureus* and *S. pneumoniae* at MIC values of 125 and 100 μg/ml, respectively. The main targets were the bacterial wall and membrane as confirmed by scanning and transmission electron microscopies, as well as fluorescence microscopy. In addition to this, significant antibiofilm activity was also observed against *S. aureus* and *S. pneumoniae* [32]. The antibacterial properties of two-fold serial dilutions of the extract and EO of *Satureja hortensis* were studied on selected bacteria important in early supragingival dental plaque formation. The dental plaque bacteria selected were *S. mutans*, *S. sanguis*, and *S. salivarius*. MIC for each bacterium was determined by broth dilution and disc diffusion methods. 30 μg tetracycline and 15 μg erythromycin served as positive controls. No significant antibacterial activity was noted with the aqueous and ME. However, significant ($P < 0.05$) growth inhibition of the test bacteria was noted with EO in comparison to the positive control. Significant ($P < 0.0001$) antimicrobial effects were observed with higher concentrations of EO against the oral bacteria than the low concentrations. 30 μg tetracycline inhibited *S. mutans* similar to 50% and 25% dosages of the EO while it was like 50% of the EO dosages for *S. salivarius*. 15 μg erythromycin tested against *S. sanguis* was less effective than 50% and 25% of the EO dosages. This effective antibacterial dose of EO from *S. hortensis* on the tested bacteria is suggestive of its application as a herbal mouth rinse [33]. *S. mutants* isolated from the saliva was exposed to the crude organic extract of Shih. The negative and positive controls were *S. mutans* with culture media only, and gentamicin treated *S. mutans,* respectively. INT chloride colorimetric assay was employed to determine MIC and MBC. The broth microdilution method was carried out to perform the time-kill dynamic assay. Polyphenols present in Neem leaf extract adhering to oral surfaces carry long-lasting antibacterial properties. They also have the potentials for synergic antioxidants in combination with bacteria, lysozyme, and red blood cells. These properties suggest their effectiveness in periodontal diseases [34]. Neem leaf extract from the Neem tree (*Azadirachta indica*) was used to assess the growth kinetics of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* under anaerobic conditions. Microdilution method was employed to determine the MIC against each bacterial strain. The visual semi-quantitative assay was used to assess the bacterial coaggregation that confers virulence. Wilkins–Chalgren anaerobic broth was used for anaerobic growth of *F. nucleatum* (ATCC 1924) and *P. gingivalis* (ATCC 33277) at 37 °C. The anaerobic growth condition was created with CO_2 (5%), H_2 (10%), and N_2 (85%). After the bacterial growth to the early stationary phase, the final concentrations of 1.5×10^{11} and 0.5×10^{11} CFU/ml were obtained for *P. gingivalis* and *F. nucleatum*, respectively. The bacterial growth kinetics were determined using an automated microtiter plate reader, at different Neem leaf extract concentrations. After aerobic growth of *F. nucleatum* at 37 °C/24 h and *P. gingivalis* at 37 °C/24 h, the optical densities were measured. Wilkins–Chalgren anaerobic broth was used to carry out broth microdilution experiments. Two-fold serial dilution range of 20–0.039 μM gallic-acid equivalents (GAE) of neem leaf extracts were used. Metronidazole at final concentrations of 2–0.004 μg/ml was the positive standard antibiotic control for anaerobic infections. The negative control was the solvent, that is 30% ethanol. 0.5 McFarland equivalent of the bacterial inocula yielding 1.5×10^8 CFU/ml, were incubated anaerobically for 72 h. MICs were then read at $OD_{650\ nm}$ in duplicates. The coaggregation of *P. gingivalis* and *F. nucleatum* was visually assessed by semi-quantitative assay in the presence or absence of neem leaf extract. *F. nucleatum* and *P. gingivalis* were washed with the coaggregation buffer and resuspended to $OD_{660\ nm} = 1$. The coaggregation buffer was composed of 1 mM Tris, pH 8.0 containing $CaCl_2$ and $MgCl_2$ (each at 0.1 mM), NaCl (0.15 M), and 0.02% NaN_3. Neem leaf extract (150 μl) was added to the equal volume of each bacterial suspension. PBS served as control. The coaggregation score ranged from 0 to 4 scale. Score 0 was attributed to visible aggregations with unchanged turbidity; score 1 was for those aggregates finely dispersed in an opaque solution; score 2 was defined for the suspended visible aggregates; score 3 for the large aggregates settling with slightly turbidity of the supernatant; and large, immediately settling aggregates, with the water-clear supernatant, were scored 4. The anti-*P. gingivalis* activity of Neem leaf extract was dose-dependent. *F. nucleatum* was not affected and there was no coaggregation of the two bacteria [34].

Use of nanoparticles

The advantages over other biological methods of using plants or plant metabolites have intrigued scientists. One of these methods is the synthesis of nanoparticles to study the mechanisms of plant absorption and bioreduction of

metal ions. The extracts from *Ocimum Sanctum* (Tulsi) leaves and its derivative quercetin was reported to be used in a study to find the role of its biomolecules in silver nanoparticles (AgNPs) formation from cationic silver.

Green chemistry was used as a separate precursor for AgNPs synthesis using Tulsi and Tulsi flavonoids under different physico-chemical conditions like temperature, pH, reactant's concentration, and reaction time. X-ray diffraction (XRD) analysis, transmission electron microscopy (TEM), and optical spectroscopy absorption, photoluminescence (PL), PL-lifetime, and Fourier transform infrared were employed to determine the size, shape, morphology, and stability of the resultant AgNPs. The inhibitory zone and MIC indices of AgNPs were determined to assess its increased antibacterial activity against *E. coli*. The advantage of the surface to volume ratio of AgNPs in comparison to their bulk counterpart facilitates AgNPs:bacterial surfaces interactions, and increases the antibacterial activity of AgNPs. AgNPs interact with sulfur and phosphorus components of the bacterial cell, leading to cell death by the deterioration of the respiratory chain and cell division. AgNPs synthesized with *O. sanctum* extract or quercetin increased antibacterial property as observed from the disc diffusion method compared to *O. sanctum* extract, aqueous quercetin, and $AgNO_3$. Compared to other samples, the inhibition halo of bacterial growth treated with AgNPs was greater. Cationic silver (Ag^+) in aqueous silvernitrate ($AgNO_3$) likewise has antibacterial impacts. Be that as it may, nanoparticles possess higher antibacterial properties than free silver particles. To calculate the MIC, the gram-negative bacteria were suspended in broth cultures containing 0–200 μg/ml of AgNPs at 50 μg/ml increments using both *O. sanctum* extract and quercetin for 24 h. The untreated cultures exhibited turbidity while AgNPs decreased the cloudiness. Ascending concentration of AgNPs brought about more clearance in turbidity. 150 μg/ml of AgNPs synthesized using both *O. sanctum* extract or quercetin resulted in a clear solution. The strategies utilized for antibacterial examination of AgNPs, propose that AgNPs with *O. sanctum* extricate and quercetin has nearly the same and equal antibacterial potential. Distinctive characterization methods revealed that biomolecules (quercetin) of Tulsi is primarily effective for the diminishment of metal particles to metal nanoparticles [35]. MIC and MBC of the crude organic extract *Xylopia sericea* fruits (OXS) were determined against some bacterial strains pathogenic to human health or causing food spoilage. A high bacteriostatic effect was noted against *K. pneumonia, S. aureus*, *E. cloacae*, and *B. cereus*. The potential of OXS in the treatment of human infection and the preservation of food is evidenced [36]. A phytochemical and antibacterial study was conducted with the isolation of six flavones from hexane extract *Trixis angustifolia*, an endemic species to Mexico against *Mycobacterium tuberculosis* H37Rv. A 25 μg/ml MIC level was determined against *M. tuberculosis* H37Rv. The intracellular mycobacterial growth was significantly inhibited by the hexanic extract concentration of 12.5 μg/ml. A fraction of hexane extract (MIC = 12.5 μg/ml) was active against *M. tuberculosis*. Pebrellin, a major flavone 1 did not exert antimycobacterial activity. Combination of the extract with pebrellin exhibited a synergistic effect indicating the presence of diverse metabolites in *T. angustifolia* [37]. In Cameroonian folk medicine, *Elaeophorbia drupifera* (Thonn.) Stapf. (Euphorbiaceae) is applied to treat some bacterial diseases like skin infections. Broth dilution method was used to determine the MIC and MBC of the methanol extract of *E. drupifera* leaves, fractions a–d, sub-fractions c1–7, and c31–35 against selected strains of Gram-negative and Gram-positive bacteria including MDR phenotypes. The leaves were fractioned and purified by column chromatography. Spectroscopy was employed to determine the chemical structures of compounds. An MIC range of 16–1024 μg/ml for EDLc3 and EDLc4 was obtained against all tested bacteria. Selective activities were noted with other sub-fractions. The fractions a and c at MIC level of 32 μg/ml, brought about growth inhibition of *E. coli* AG100, and *E. aerogenes* ATCC13048 and EA298 respectively. At 16 μg/ml concentration, the sub-fractions were active against *S. aureus* MRSA11 with c3. *Providencia stuartii* NAE16 was affected at 8 μg/ml of fraction c33. MIC of <100 μg/ml of fraction c3 was recorded for all the tested bacteria. *E. drupifera* was a potential phytomedicine against MDR bacteria with the sub-fraction c3 being more active than all other isolated compounds [38]. *Propionibacterium acnes* brings about an inflammation that induces production of IL-6 and oxidative stress. MIC of *P. acnes* and *S. aureus* mixed with BHI broth or MH was determined. *P. acnes* were anaerobically grown for 3 days in BHI medium at 37 °C. PBS was used to obtain approximately 1×10^8 CFU/mL of *P. acnes* by adjusting the bacterial suspension to an optical density of 0.14. *S. aureus* was grown aerobically at 37 °C in MH medium and the bacterial density was adjusted photometrically to 0.08 equivalent to 10^8 CFU/ml in PBS. Five milliliter of two-fold concentrated brain heart infusion (BHI) or MH medium were seeded with 50 μl suspension of *S. aureus* or *P. acnes*. 100 μl of this suspension containing 5×10^4 bacteria was added per well of a microtiter plate followed by addition of equal volume of sterile water with hop extract. Two-fold serial dilutions of DMSO dissolved hop extract were made to obtain 0.19–50 μg/ml concentrations. Clindamycin served as positive control and hop extract solvent, DMSO, and water were negative controls. The samples of *P. acnes* and *S. aureus* were then incubated under anaerobic and aerobic conditions at 37 °C, respectively. The lowest concentration of the hop inhibiting the bacterial growth was defined as MIC. Agar diffusion test was employed to determine the antibacterial activity of several gel preparations. The incubation of *P. acnes* in BHI was carried out under anaerobic conditions at 37 °C for 3 days to achieve

confluency of about 10^4 CFU/ml. *P. acnes* or *S. aureus* was spread on the MH or BHI agar plates, and allowed to dry. The gel preparations were placed in the 3 mm diameter holes aseptically bored on the agar plates. *P. acnes* plates were incubated at 37 °C for 3–4 days under anaerobic condition while *S. aureus* was aerobically incubated at 37 °C for 24 h. An acne-gel containing *Boswellia serata* extract with retinol, and the botanical gel containing hop extract was used while the gel base served used as a placebo. The positive control was a gel preparation with clindamycin and benzoyl peroxide on 1:5 (w/w) ratio. The diameter of the bacterial growth inhibition zone was measured to determine the antibacterial activity. The susceptibility to the hop extract of *S. aureus* and *P. acnes* was tested by broth microdilution. The MIC levels of 9.4 and 3.1 μg/ml were determined for *S. aureus* and *P. acnes* respectively [20]. Antimicrobial activities of herbal extract (acorn) against intestinal tract pathogens were studied *in vitro* and *in vivo*. Current antibiotics were used as standard compounds for comparison of the results of the antibacterial effect of herbal extracts. MICs at 5, 10, 10, 15, and 15 μg/ml levels were obtained for ethanolic extract of acorn herb (Jaft) against *S. typhi*, *E. coli*, *K. pneumoniae*, *P. aeroginosa*, and *S. aureus* respectively. The rats were infected with *E. coli*, *K. pneumoniae*, *S. typhi*, and *P. aeroginosa* and treated with acorn extraction. The treated rat groups survived while the control groups died after 5 days [39].

Chemical composition of essential oils

The most dominant compounds of *Artemisia herba-alba* oil are oxygen-containing monoterpenes, namely alpha-thujone (22.9%), beta-thujone (25.1%), camphor (10.5%), and 1,8-cineole (20.1%) [40]. The chemical composition of the ME and EO of the leaves of *Anthemis stiparum* subsp. sabulicola was analyzed, and the total phenolic and flavonoid contents, anticholinesterase, antioxidant, antibiofilm, and antimicrobial activities were determined. Seventy-two compounds (99.02%) were determined by GC–MS and gas chromatography-flame ionization detection (GC-FID) from the aerial parts of *Anthemis stiparum* subsp. Sabulicola. Germacrene D, t-cadinol, camphor, spathulenol, and isoamyl salicylate were predominant chemical composition at 11.13, 11.01, 6.73, 6.50, and 6.45% levels respectively. Flavonoid and total phenolic contents of the ME were determined spectrophotometrically. The pyrocatechol equivalents and quercetin equivalents of total phenolic and flavonoid contents of ME were about 13.6 and 5.9, respectively. Antimicrobial activity of the ME of *A. stiparum* subsp. sabulicola (Pomel) Oberpr. was better than its EO. MIC value of 1.56 mg/ml was obtained against *B. subtilis* (ATCC 6633), and *S. aureus* (ATCC 25923) [41]. Carvacrol, E-caryophyllene, and caryophyllene oxide constituted 48.9, 10.8, and 7.7% of the chemical composition of *L. coronopifolia* EO, respectively [42]. The biological activity of EO from *Poliomintha longiflora,* suggest that antimicrobial and antioxidant compounds can remain in *P. longiflora* hydrosol and waste solid residues [43]. Understanding the mechanism of action of the compounds implicated in the bioactivities of the crude extracts requires further investigations [17].

Time-kill dynamic assay

Broth microdilution method was used to perform a time-kill dynamic assay of the crude extract of Artemisia herba-alba (Shih), and gentamicin. 1×10^6 CFU/ml suspension of *S. mutans* were treated with 1/2 MIC, MIC, and MBC of the samples and incubated at 37 °C for 0–960 min. DMSO was used at 2.5% final concentration. 2.5% DMSO and the inoculum served as the control. 10× serial dilutions were prepared from 50 μl of the sample mixture taken at 0, 30, 60, 120, 240, 480, and 960 min of incubation. 25 μl suspensions of each dilution was cultured on the MHA for 24 h at 37 °C. The CFU/ml was plotted against time to build the time-kill curves [30]. Antifungal and anti-inflammatory doses of *Artemisia herba-alba* oil were not detrimental on various mammalian cell types [40]. The ethanolic extract of *Rhodomyrtus tomentosa* leaves was studied for its antibacterial effect against *S. aureus*, the causative agent of bovine mastitis. Microdilution method was employed to determine the MICs of the extract and a pure compound isolated from rhodomyrtone. MHB was adjusted to 256 μg/ml final cation concentration. A maximum of ≤0.5% DMSO concentration was used. The agents were added separately, and two-fold serial dilutions were prepared in microplates. The same volume of the bacterial suspension at $\sim 1 \times 10^6$ CFU/ml concentration was then added to each well and incubated for 18–24 h at 35 °C. All assays were controlled by sterility and bacterial growth. The quality control reference drugs were vancomycin and pirlimycin hydrochloride. The lowest concentration yielding no detectable macroscopic growth was defined as the drug MIC. To determine MBC, the TSA plates were streaked with 10 μl from the MIC and higher concentrations and incubated at 35 °C for 18 h. The concentration causing 99.9% death of the initial inoculum was defined as MBC. A two-dimensional microdilution checkerboard assay was performed against *S. aureus* Newbould to determine MICs of the extract of rhodomyrtone in combination with oxacillin, amoxicillin, pirlimycin, penicillin, ampicillin, gentamicin, ciprofloxacin, kanamycin, ceftiofur, and oxy-tetracycline. The X-axis included serial dilutions of the extract, or rhodomyrtone, and the antibiotics on the Y-axis. The bacterial suspension of $\sim 1 \times 10^6$ CFU/ml was added at an equal volume to each well containing the antibacterial agents and incubated at 35 °C for 18–24 h. The lowest concentration of the drug, or drug combination showing no detectable macroscopic growth was defined as MIC. The antibacterial effect was calculated using the fractional inhibitory concentration (FIC) index:

FICA = MIC combination/MICA, FICB = MIC combination/MICB, $\sum$FIC = FICA + FICB. The interpretation of FIC index was $\sum$FIC values of ≤0.5; >0.5–1;

>1–4; and ≥4, for antagonism. Synergism, additivity, and indifference respectively.

S. aureus Newbould is wild-type and its isogenic Δ*hemB* mutant represents SCV phenotype. The bactericidal activity of the extract and rhodomyrtone were assessed using Time-kill experiments. McFarland standards were used to prepare the bacterial inoculum of $\sim 10^6$ CFU/ml by optical density assay which was further confirmed by plating on agar. 0.5×, 1×, 2×, and 4× inoculum concentrations of their respective MIC value, were grown in CAMHB supplemented with the ethanolic extract of the plant or rhodomyrtone. The bacterial culture was monitored for growth over 24 h. A 10 μl aliquot from 10-fold serial samples dilutions taken at various time intervals were spread onto TSA plates to enumerate the CFU. Log_{10} CFU/ml versus time curve was plotted to analyze the bacterial growth kinetics. Strong bactericidal activity was shown by time-kill kinetics for bacteria treated with double MIC of both the plant extract and rhodomyrtone within 4 h. Promising in vitro antibacterial activities of the extract were noted. In-vivo experiments are necessary to consider the findings as antibiotic alternatives in dairy farms [19].

Discussion

Recently, the increasing resistance of bacteria against antibiotics and other chemical agents has attracted attention to focus on natural products characterized by antimicrobial properties. On the other hand, the side effects of antibiotics are yet another concern. In the meantime, special focus was made on medicinal plants. The choice of medicinal plants is mostly based on their traditional use or experimental documents. Such research is very good for creating evidence, but it must be done scientifically using standard methods. Invalid testing methods will produce confusing information. One of the main sources for this category of research is the use of CLSI documents, and other reference books are less commonly used. EOs are a complex mixture of organic volatile and odoriferous compounds. A good number of publications highlighting the antimicrobial properties of EOs are available worldwide. These publications indicate a wide range of EOs with antibacterial, antifungal, and antiviral activity. EOs were shown to effectively act against drug-resistant microbial strains. In fungal pathogens, EOs disrupt ATP assembly, establish membrane potential across the cell wall, and damage the cell membrane. The electron transport system pathway is interfered with by EO causing the disintegration of the mitochondrial membrane. The cellular architecture of the bacterial pathogens is primarily destabilized by EOs, hampering the membrane integrity, and disrupt cellular energy production and membrane transport. The cellular components leakage and subsequent loss of ions are brought about by membrane rupture induced by EOs [44]. The important purpose of this review article is to study the assessment methods of the efficacy of the antibacterial compounds from natural sources. Numerous articles study the effects of different types of extracts that have been extracted in different forms and different methods have been used for this purpose.

Some articles do not mention the location and time, nor do they specify at what stage of the plant growth the sample was collected. More importantly, the identification of genus and species has not been confirmed by reference organizations. Because the chemical composition of a plant is very different in terms of production time, location, and growth stage, it can contain different compounds and greatly affect the results. Various studies of the chemical analysis confirm this. In many cases, the species is misidentified. In some articles, for example, the species of Thymus has been confused with Zataria. Very often the Latin and local names are confusingly used. Another drawback in this regard is the indigenous nature of the plant in the country where a study is carried out. In some cases, this word is used incorrectly due to a lack of botanical knowledge or expertise in the definition of indigenousness. Therefore, it is commonly observed that in some countries, a specific plant is mentioned as a native plant of their region in the articles. While obtaining a voucher number, can solve many of these problems. Also, in some studies, plant samples are obtained from the grocery or local markets which is an additional problem. Since plants are collected in the vicinity of the soil, the necessary attention should be paid to the collection with the least contamination risks. Contamination includes soil particles, microorganisms, spores, and parasitic cysts. The presence of toxins and the use of fertilizers may also be effective as a result of the work and the procedure. Following the principles of sampling and correct transfers can help reduce pollution. The extraction of EOs is done at high-temperatures or by using solvents. High temperatures or most solvents carry antimicrobial effects rendering the EOs sterile. In suspicious cases, it is better to cultivate the sample. If contaminated, other methods can be used to suit the physicochemical properties. Including the use of 0.2 or 0.4 μm filters. The limitation of the latter method is that large particles in the extracts accumulate on the filter and prevent the easy passage of liquid. This problem becomes severe when the extract is mucoid. It is always advisable to first use a filter with larger pores to separate the debris and then use the filter to sterilize. The above is less common for EOs because many of these problems are solved during the extraction process.

Another step is to dry the plant. This is usually done in several ways. The most common method is drying in the open air in the shadow or under the sun. Drying is also done in the oven. However, it should be noted that the rate of drying is very important. It can be very effective if work is based on the weight/volume (w/v). The method

of drying with an oven should also take into account that if the temperature of the device is high, it can change the nature of the compounds. The next step is the identification of the chemical composition of the herbal extract or EO. This practice is different in different studies. At this stage, some probable problems like the choice of an appropriate solvent are not considered. The solvent is a liquid in which the dry powder of the plant dissolves and facilitates further steps of the extraction of EOs. Solvents are usually divided into polar and nonpolar categories. It is selected based on the nature of the plant and the separation of the active ingredients and the next steps.

The followings are the characteristics of an appropriate solvent:

(1) Preferably it has no antimicrobial effect or it can completely be removed from the product after extracting the plant product.
(2) It is used in small quantities.
(3) Do not create multiphase mode
(4) It should be easily removed in the next steps.
(5) The solvent should not create interfering color when the quantitative estimation of the study is planned to use a spectrophotometer or colorimeter.
(6) It has the least effect on changing the nature of the plant extract or EO compounds

In most articles, the widely used solvents are water, methanol, ethanol, DMSO, Tween, acetone, acetonitrile, etc. In general, the use of inappropriate solvents causes erroneous results. A stock solution that contains the highest concentration of the EO or extracts in the solvent (usually 512 μg/g or μl/ml), should be made. Water is the most favorable solvent and should be used when possible. The stock solution can be prepared on the weight/volume (w/v) or volume/volume (v/v) basis. In most studies, the sequence of concentrations must be established following the preparation of the stock solution. At this stage, the volume should be weighed as much as possible. The final concentration range should be 512–0.125 μl/ml (or μg/ml). Another important point to note here is to adjust the concentration range to avoid misleading results unless another method is used for appropriate reasons. In some studies, especially in the v/v method, the reversed path has been employed. Technically, this method causes problems. To make it easy to work, it is recommended to weigh the extract or essential oil and then prepare the concentration range by w/v. Most studies refer to the guidelines published by the Clinical Laboratory Standard Institute (CLSI) as their reference in testing the antimicrobial effects. These guidelines are meant for antibiotics and do not refer to medicinal plants or their extracts. The methods mentioned in CLSI are the result of thousands of experiments for each of the antibiotics to determine the breakpoints. These breakpoints are important in determining the amount of resistance and sensitivity and are based on the halo diameter, the lack of growth in the diffusion disc method, or the amount of concentration in the MIC methods. Failure to grow in the disc method is diffusion or concentration in MIC methods. Finally, using the results of the study of resistance and sensitivity, in many clinical samples, the breakpoints are determined with pharmaco-kinetic assays. The amount needed to create the necessary concentration in the body (*in vivo*) is determined thereby. In some other studies, the CLSI guidelines have often been used in a modified form that in many cases leads to seriously erroneous results. The following are the bugs in such articles.

Disc diffusion

In this method, the antimicrobial material is loaded on sterile paper discs. The discs are then placed on the agar plate surface to measure the zone of growth inhibition. There are several drawbacks to this method. These include solvent, the size or diameter of the paper disk, the amount of loaded compound, whether or not drying time is given, and how the discs are placed. One of the most important points that are not considered in most of such studies is the rate of penetration of the test compound in the culture medium. This diffusion depends on the molecular size and the type of electric charge. This is the reason for the different results obtained with the effective diameter of the halo of antibiotics, the amount of propagation in the culture medium, the breakpoints. Even in the recent documents, different results are given for the same antibiotic. As the effective diameter of the inhibition zone for EOs is not yet known, therefore, it is not possible to determine the diameters of the growth inhibition, resistance, or sensitivity. To solve this problem, the steps mentioned in CLSI should be considered and then appropriate changes should be applied for plant extracts or EOs. In some other articles, has been the results are compared with antibiotics which are invalid results. In general, it can be concluded that the disc diffusion method can be more useful for primary screening. However, the same method should research with a single and unique procedure to arrive at a correct conclusion.

Well diffusion

This method is almost obsolete, however, it can be seen in some articles. In this method, the amount of antimicrobial effect is investigated by creating a well and filling it with an extract, or EO. In addition to the disadvantages of the disc diffusion method, the main drawback is that in some cases the boring of an agar culture is performed after the bacterial inoculation on the surface of the medium. In this case, the bacterium enters the well and affects the result.

Agar dilution

This method is one of the valid standard methods and is widely used in the study of antimicrobial effects. In this

method, by creating the desired concentrations in a suitable agar culture medium, such as Mueller Hinton agar, the antimicrobial effect is investigated. One of the good features of this method is that if the bacterial inoculation is CFU/spot at the same time, the antimicrobial effect of a substance can be exerted on several bacteria. In this method, the main limitation is the need for a large amount of extract or EO. However, MBC cannot be determined by this method.

Broth dilution method

This procedure is performed in two ways of macro dilution and microdilution methods. These methods are similarly implemented, except that the macro dilution method is performed in large volumes (2 ml in a tube) and the micro method is performed in small volumes (100 µl in each well of 96-well microplates). In terms of the standardization of the work, the macro method is preferred. However, due to the need for a high amount of materials and handling difficulties of the tubes, the tendency to go this method is less. In these two methods, the values of MIC and MBC are obtained by creating a concentration or a dilution range. In this method, too, most researchers refer to CLSI instructions. The CLSI has no specific method for medicinal plants. Researchers are commonly used to measure the antimicrobial effects of medicinal plants, such as the disc diffusion method, by modifying the broth dilution method mentioned in the CLSI. In this case, several problems are encountered. Among the main problems, the followings predominate.

- Most investigators are not microbiologists nor do they have a microbiologist consultant.
- Failure to observe the concentration range or dilution correctly.
- Inaccuracy in observing the ratio of the concentration of the culture medium and the inoculated suspension
- No attention is paid to the color changes resulting from the addition of extracts or essential oils.
- The antimicrobial role of solvent used in preparing the concentration range or dilution is ignored.
- Lack of full knowledge of MBC determination methods.

Other important points

One of the important points in most articles is the lack of a study plan on toxicity, mutagenicity, carcinogenesis, and teratogenicity. Determination of chemical composition can greatly help in finding the active ingredients. This is usually done with GC, GC/MS, HPLC, or chromatography methods. Some studies have reported the above information, however, a few articles are examining the antimicrobial effects of extract simultaneously with the active ingredients. Such studies facilitate the identification of the active chemical compounds and hence help standardize the methods. Comparison of the antimicrobial effects of medicinal plants with antibiotics is not justified. This is because the breakpoint of medicinal plants is not specified. However, studying the synergistic effect of medicinal plants with antibiotics can be useful and provide good information. Experiments such as antioxidant, anti-inflammatory, immune-boosting, etc. can also be strengths of the articles. Lack of animal models is another flaw found in most articles. In other words, to determine the effectiveness of the medicinal plant, it is necessary to test its therapeutic effect in animal models. To perform this test, it is necessary to prepare a formulation. The preparation of this formulation requires the cooperation of multidisciplinary experts like a pharmacist, pharmacologist, and animal physiologist. To standardize experiments, the use of standard microbial strains is very useful, especially when clinical isolates are included in the study. Serious attention should be paid to statistical calculation methods. Usually, such studies should be performed in three triple repetitions, and then the results should be interpreted using the appropriate formula. Broth dilution method can be used to perform the time-kill dynamic assay [30]. Synergic interactions, inhibition of biofilm formation, using nanoparticles, and determination of chemical combination can also be useful in such research.

Suggested methods for unifying study protocols

It is very important to provide a single method, and this method should be based on a valid reference. Since there is no direct reference to this case in any of the sources, the documents like M11-A and M23-T3 Vol. 18 No. 5 of the NCCLS may be referred to as major references with reliable modifications applicable to EOs as proposed in this review to unify the methods. In addition to these references, other additional documents have been published under the title CLSI [45–47]. Since this document is quite an appropriate reference, it has been used as the basis for the recommendations in this article. The following steps are recommended to evaluate the antibacterial effect of herbal extracts or EOs:

Selection of a solvent

First of all the antimicrobial effect of the solvent should be examined. The best method for this test is the disc diffusion method. To perform this experiment, several standard bacteria should be selected from different groups. A bacterial suspension should be made with the turbidity of 0.5 McFarland standard, and uniformly inoculated on a suitable culture medium (usually Muller-Hinton). 10–15 µl of the solvent is added onto sterile blank discs of 6 mm diameter placed on the preinoculated culture medium, and incubate at 37 °C overnight. The solvent will not be considered suitable if it inhibits the growth, and should be replaced by another solvent.

A stock preparation of essential oils

The stock mixture should be prepared after determining the appropriate solvent. For this purpose, mix equal weight

or volume of the extract, or EO with solvent and mix well. If it dissolves well, the amount of solvent can be reduced to a minimum volume. Alternatively, if the extract or essential oil does not dissolve well in the solvent, the solvent volume should be increased until it completely dissolves the extract or the EO. Under these conditions, the resulting mixture should be stored refrigerated in the dark as a stock dilution. Some extracts precipitate in the refrigerator, and they should not be refrigerated. It should also be noted that they should be used as soon as possible.

Determination of antimicrobial effect by disc diffusion method

In this method, the following steps must be performed:

Preparation of a 24-h culture of the test bacteria and standard strains in an appropriate liquid medium (in most cases Muller-Hinton broth). The suspension concentration is then adjusted to obtain 0.5 McFarland standard level by adding the culture medium. This will yield a bacterial number of about 10^8 CFU/ml. A swab may be used to spread inoculate the bacterial suspension onto the solid culture medium. The plates are left on the laboratory bench until the moisture is absorbed. 10–15 μl of serial double dilutions of the extract, or EO is loaded on the sterile blank discs. Usually, the final concentration range in this method is within the range of 512–0.125 μg or μl per ml. The plates are kept at room temperature for 1–4 h after loading until the EO or extract is completely absorbed in the disc. The discs are then placed on the culture medium at a suitable distance from the edge and each other. The distance should not allow the growth inhibition zones to merge or touch the edge of the petri dish. Sterile micropipette tips are used to gently apply pressure to remove the gap between the disc and the agar. The plates are incubated in an inverted position at 37 °C overnight. The diameters of the growth inhibition zones are measured with a caliper or a ruler. This method is useful for the initial screening of the antibacterial effect of medicinal plant extracts or oils.

Agar dilution method

This method is one of the most appropriate methods in determining the antimicrobial effects of various substances. Despite its suitability, a considerably low number of reports are available to the use of this method. In this method, various concentrations of an extract or oil, are added in a suitable culture medium containing agar and the antimicrobial effect is investigated. Besides, in this method, the MIC can simultaneously be determined. The simultaneous study of the antimicrobial effect of one substance for several bacteria is another feature of this method. The procedure is as follows.

In each of 10 tubes, 9 ml of suitable agar medium is added, and after autoclaving the tubes, the temperature is let to reduce to 45 °C. While the medium is in a liquid state, one mL of the mixture containing the antimicrobial agent is added, so that the desired final concentration is achieved. The tubes are vortexed and poured into a petri dish to achieve a 5 mm diameter of the culture medium. The plates are allowed to solidify and are then inoculated with a 24-h old culture of the desired bacterium. The bacterial suspension of 5×10^5 CFU/ml concentration should be subcultured. MIC is the minimum concentration of the plant extract or EO that inhibits bacterial growth. Haze formation or formation of several colonies is considered a lack of growth. Alternatively, a modified method can also be used. In this method, 1–2 μl containing 10^4 bacteria are inoculated in a circle of 1 cm diameter (CFU/spot). In this method, several bacteria can be tested on every plate to save time and materials. The formation of Haze mode or the presence of a few colonies should be considered as negative for bacterial growth.

Broth dilution method

The broth dilution is the main method to determine the antimicrobial effect in most articles. One of the good features is obtaining MIC and MBC values. This method is followed in two forms of micro and macro dilutions. Macro dilution is carried out in the test tube while microdilution is performed in less volume in 96-well plates.

Macro dilution method

A 24-h old bacterial suspension is prepared at 1×10^6 CFU/ml concentration. One milliliter from The above suspension is added to each of the 10 test tubes. A range of two-fold dilutions is made from the antimicrobial agent. One milliliter of each dilution is added to each of the nine tubes. One mL of the liquid medium without the antimicrobial agent is added to one tube. Immediately 10 μl is inoculated from the control sample on the surface of the plate and the plate is incubated at 37 °C. The number of colonies grown after 18–24 h in the control well is counted by successive dilutions, and the total number of bacteria per unit volume for the control sample is set at time zero. The test tubes are observed for growth after 18–24 h. The highest dilution of the test material rendering no turbidity or bacterial growth is recorded as MIC. To determine the MBC, the number of bacteria should be counted from all clear tubes by the serial dilution method. The minimum concentration or the highest dilution causing 99.9% of death is defined as MBC.

Although this method has been used extensively in the literature, some technical mistakes are evident. The very first mistake is that the bacterial suspension was prepared in solvent or water and was then added to the tubes. In this case, the final concentration of the culture medium is reduced by half causing a false result. If the bacterial suspension cannot be prepared in the culture medium, a double concentration of the culture medium should be prepared in the tubes until the final concentration of the culture medium is achieved after the addition of the bacterial suspension. Development of color or turbidity by the extract or EO should also be carefully considered.

This problem can lead to erroneous results. To solving this problem, the following method may be used:

Using a spectrophotometer, an appropriate optical density may be determined for the control sample to use as a blank. The turbidity of the test tubes is then measured at the obtained wavelength. The lowest concentration where the turbidity was significantly reduced is considered the MIC and is determined as mentioned earlier with the MBC.

Micro dilution method

This method is performed as in the macro dilution method. The only difference is the volume. The test is performed with 100 μl in a 96-well plate, and measured with an ELISA reader.

Recommendations

(1) Include animal models in the studies where applicable.
(2) Pharmacological examinations of the extract and EO should be performed simultaneously.
(3) The active ingredients should be studied for their antibacterial effects.
(4) Clinical trials may be done after having the study plan approved by ethical committees.
(5) Appropriate and relevant statistical methods should be used in each experiment.
(6) Use of weight in volume (μg/ml) is recommended instead of the volumetric unit for EOs or extracts (μl/ml) dissolved in solvents.

Conclusion

Many research documents are available on the antibacterial properties of medicinal plants. Lack of a widely accepted or universally approved method, based on a standard guideline, has led researchers to perform their experiments using a variety of methods and with multiple drawbacks. In the present article, only the dimensions of the antibacterial effects of plant extracts or essential oils have been discussed. Of course, problems still exist in other aspects of the research in the antibacterial properties of herbs or herbal products. For example, when the extracts or EOs affect the growth of pathogens, the problems become more complex. Because each study requires its appropriate method to be followed. In various categories of publications, we can find some experiments that are not only necessary but also misleading to discrepancies or misconceptions. For example, combining and comparing the effectiveness of an antibiotic is useful when the synergistic effect between these two is studied. This concept does not apply to a comparative study between an extract or an EO with an antibiotic. The use of standard microbial strains to standardize and replicate the experiment is necessary so that other researchers can evaluate its accuracy. Many studies are conducted only on clinical isolates where many interfering factors exist and lead to misinterpretation of the findings. In such circumstances, the standard microbial strains may be included in the study as the experimental positive control. In general, based on the contents and analysis of the published articles, it is clear that a study plan on the antibacterial effect of a plant should include EOs rather than the extract. This is mainly because the EOs compositions are detectable and the results are more reproducible to standardize.

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Conflicts of interest

There are no conflicts of interest.

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