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In vitro Methods to Study Antioxidant and Some Biological Activities of Essential Oils: a Review

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Abstract: As essential oils (EOs) represent a new source of efficient and safe agents for health nowadays, the present review brings together the *in vitro* methods widely used to evaluate the antioxidant and some biological activities especially, antidiabetic, anticancer, antimicrobial, and anti-inflammatory activities of EOs, in order to valorize these EOs and to highlight their potential benefits. Moreover, each method cited is along with its aim, principle, advantages and limitations, experimental protocols, and notes. Hence, this review will help researchers working on EOs, to save time while accessing this summary document on the one hand, and on the other hand, it will contribute to scientific approval of *in vitro* antioxidant and biological effects of EOs for future useful purposes.

Keywords: essential oils; bioactive molecules; *in vitro* methods; biological activities.

1. Introduction

Currently, therapeutic drug failure (DTF) becomes a major public health challenge. This is due to the inefficiency of synthetic drugs, especially synthetic antioxidants [1,2], antibiotics [3], chemotherapeutic agents [4], anti-inflammatory, and antidiabetic drugs, which also cause serious side effects [5] such as liver and kidney toxicity, hypoglycemia, heart problems, and gastrointestinal reaction [6]; thus, an urgent need to develop new, better, and safer natural agents to fight against DTF[7].

Indeed, natural products are nowadays considered as a potential source for discovering new and efficient agents without side effects, especially EOs, from aromatic and medicinal plants[8–10]since they are used in healing therapies, in particular, aromatherapy [11]; a natural way to heal a person's body, mind, and soul through EOs [12,13].

Besides, EOs have a complex and variable composition resulting in many pharmacological properties[11,14,15], which allows them to be involved in the management of various diseases, such as cancer, microbial infections[16–18], diabetes, and chronic inflammation [19]. They can also combat oxidative stress [19], eventually leading to the above-mentioned diseases and affecting food quality [20].

Therewith, it is necessary to target the right methods and techniques to evaluate the biological activities of EOs, whether *in vitro* or *in vivo*. Except that *in vitro* methods are simpler in their applications which makes them more feasible. They provide reliable results in a short time and avoid the use of animals [21].

In this context, this review aims to collect and describe in detail the most common *in vitro* techniques for evaluating antioxidant, antimicrobial, anti-inflammatory, antidiabetic, and anticancer properties of EOs. The present review could also serve as a guiding document for researchers working on EOs. This study reports for the first time the different *in vitro* techniques aimed at evaluating the biological activities of EOs.

2. Evaluation of antioxidant activity

2.1. 2,2-DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay.

The DPPH test measures the ability of an antioxidant compound to act as a hydrogen donor or free radical scavenger. It is based on reducing the violet DPPH to stable pale yellow DPPH molecules in the presence of an antioxidant agent.

The DPPH test is simple, rapid, and reliable; however, it is sensitive to some Lewis bases and requires organic solvents and non-physiological radicals [22–25]. The DPPH test protocol is schematized below (Figure 1), according to the method described by Shimamura *et al.* [26].

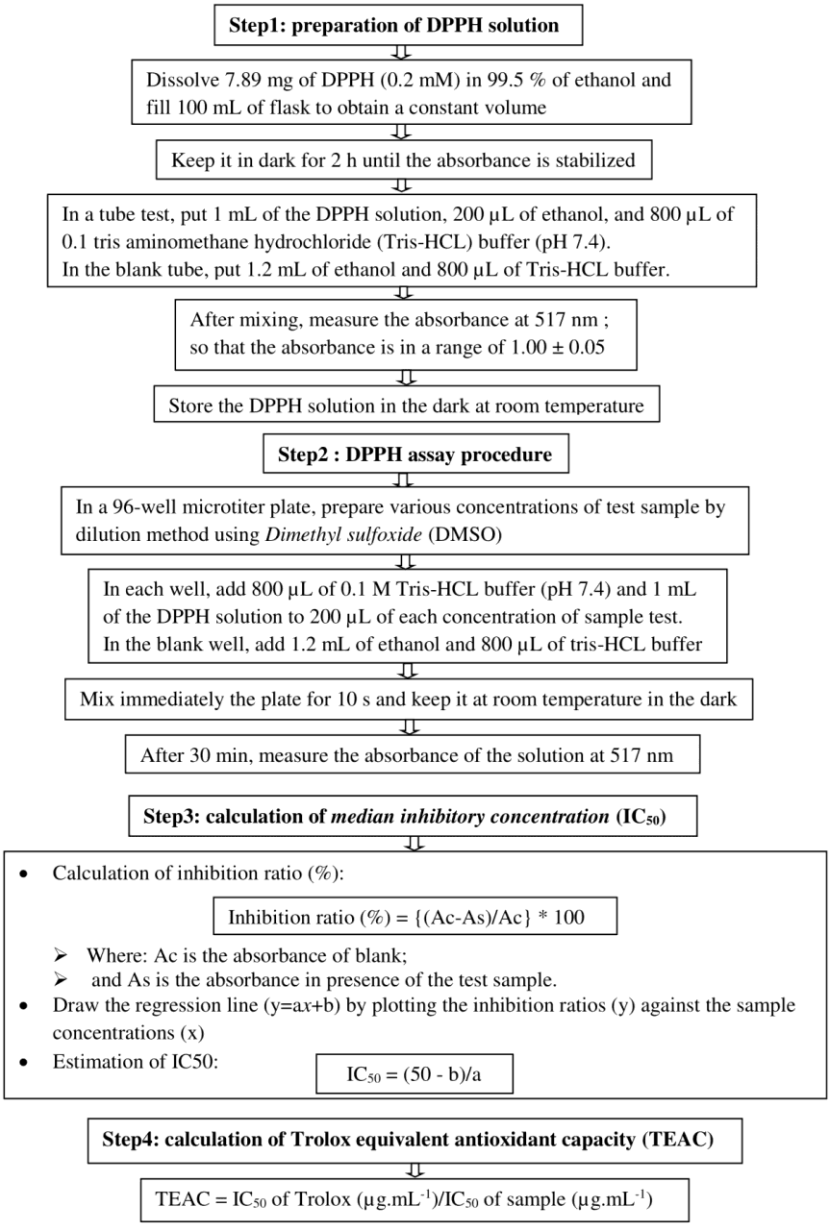

Figure 1. Experimental protocol of DPPH assay to assess the antioxidant activity of EOs.

Note: The DPPH radical scavenging activity of the test sample is expressed as the TEAC. The higher TEAC means the higher DPPH radical scavenging activity. Trolox is used as positive control; its IC_{50} is determined by DPPH assay. The assay is repeated three times [26,27].

2.2. ABTS (2,2'-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid)) assay.

The ABTS or TEAC assay measures the ability of an antioxidant to stabilize the ABTS radical cation (ABTS $\cdot^+$) by an electron transfer mechanism. The ABTS $\cdot^+$ is a green-blue chromophore produced through a reaction between ABTS and potassium persulfate ($K_2S_2O_8$). Indeed, the degree of discoloration is proportional to the ABTS $\cdot^+$ inhibition [29].

The ABTS assay is rapid, easy, and useful for both hydrophilic and lipophilic compounds. It can also be studied over a wide range of pH values. However, it is limited by the use of non-physiological radicals and the careful monitoring of time intervals [13,29]. The ABTS test protocol (Figure 2) is shown below, according to the method described by Kokina *et al.* [29] and Zhou *et al.* [30].

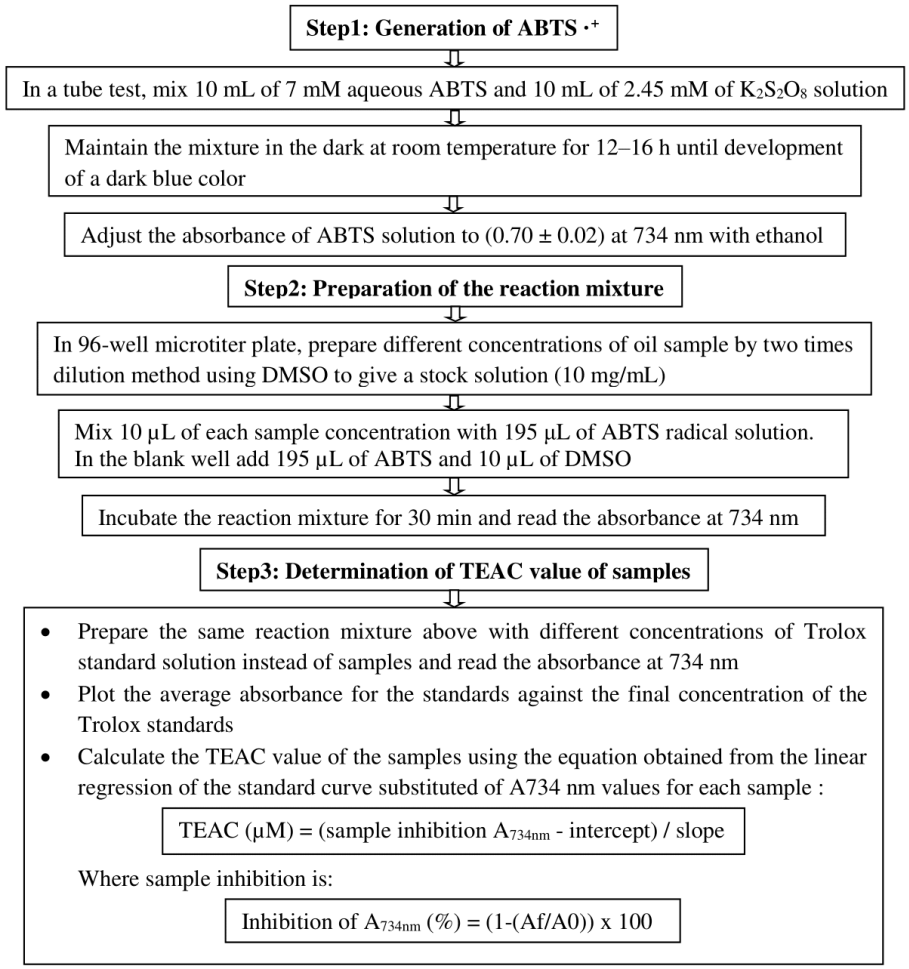

Figure 2. Experimental protocol of ABTS assay to assess the antioxidant activity of EOs.

Note: The ABTS $\cdot^+$ chromophore has three absorption maxima at wavelengths of 645, 734, and 815 nm. Trolox is used as standard, and results are expressed in mmol Trolox equivalents per liter of EO. All measurements are performed in triplicate [29–30].

2.3. FRAP (Ferric reducing antioxidant power) assay.

The FRAP assay is based on the reduction of ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to blue-colored ferrous tripyridyltriazine complex (Fe^{2+}-TPTZ) at low pH through electron-donating antioxidants [31].

The FRAP assay is simple, inexpensive, fast, and reproducible. Despite that, this assay is carried at non-physiological pH and does not measure all groups containing antioxidants, such as the sulfhydryl group; thus, it cannot necessarily reflect the entire antioxidant activity of a test sample [25]. Below, the FRAP test protocol (Figure 3) described by Benzie & Strain [32] and modified by Pulido *et al.* [33].

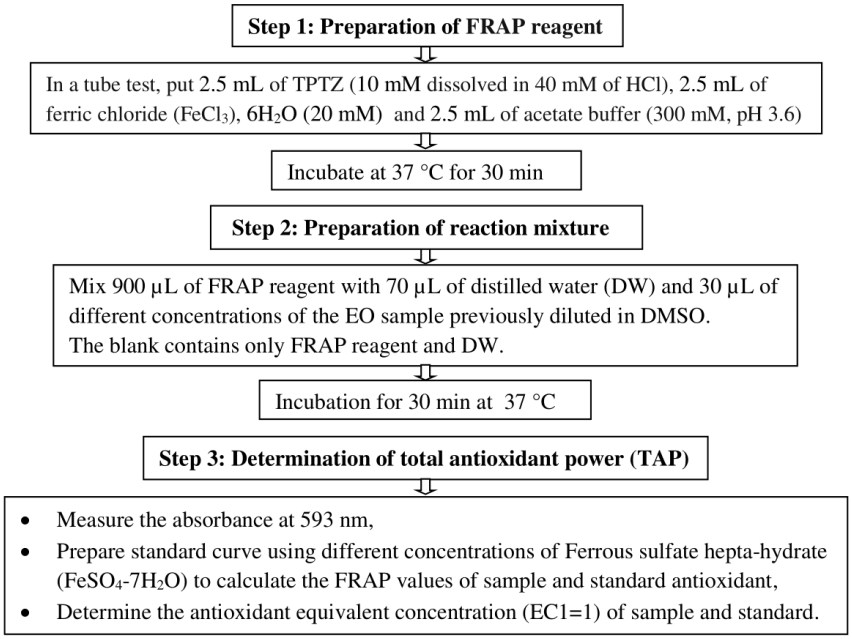

Figure 3. Experimental protocol of FRAP assay to assess the antioxidant activity of EOs.

Note: EC1 means the concentration of the antioxidant having a ferric reducing power equivalent to that of 1 mM of $FeSO_4$-$7H_2O$, determined by the corresponding regression equation. The results are expressed in µM equivalent to $FeSO_4$-$7H_2O$ [34].

3. Evaluation of anti-inflammatory activity

3.1. Nitric oxide (NO) production assay.

The measurement of NO production is a method to assess the anti-inflammatory properties of EOs through measurement of the NO· accumulation in a culture medium using the Griess reaction; thus, a low concentration of NO· reveals an anti-inflammatory activity of the sample tested [35].

The NO production assay is greatly sensitive and reproducible with rapid analysis time. However, it is not suitable for monitoring NO in real-time. Moreover, careful control experiments must be performed to distinguish basal Nitrite (NO_2^-) levels from those that arise from actual changes in NO concentration [36]. The assay protocol, according to Borges *et al.*[35], is described below (Figure 4).

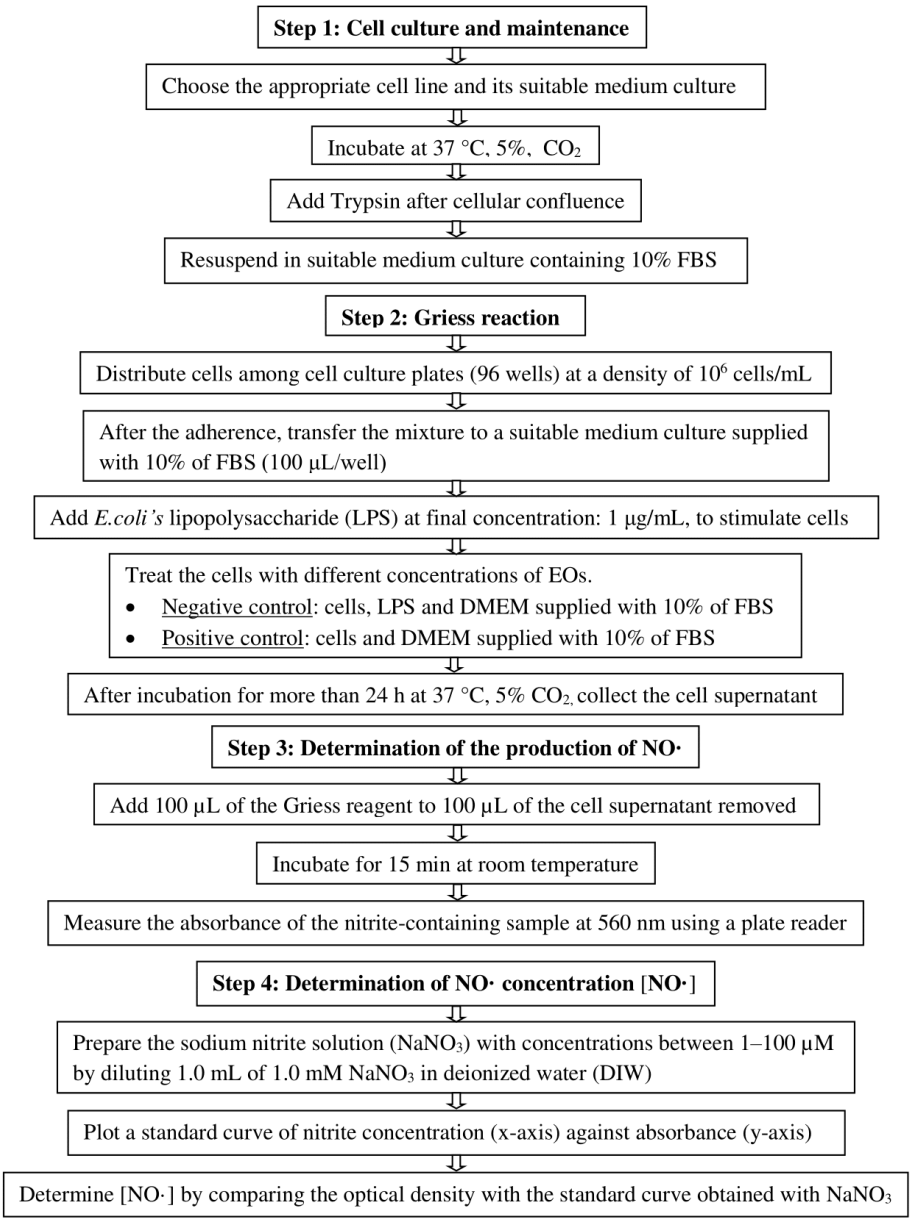

Figure 4. Experimental protocol of NO production assay to assess the anti-inflammatory activity of EOs.

Note: The cell lines widely used are Macrophages J774A1 and RAW 264.7 cells [35]. The DMEM represents the suitable culture medium containing the necessary nutrients and growth factors for cell lines. Griess reagent is prepared by mixing equal volumes of stock solutions of chloride-1-ethylenediamine ($C_{12}H_{16}Cl_2Na_2$) dissolved at 0.1% in phosphoric acid (H_3PO_4) 5% and sulfanilamide ($C_6H_8N_2O_2S$) dissolved at 1% in H_3PO_4 (5%). All experiments are performed in triplicate [37,38].

3.2. Cyclooxygenase (COX) inhibition assay.

There are two isoforms of COX in mammals; a constitutive form (COX-1) and an inducible form (COX-2), which is responsible for the dramatic increase of prostaglandins (PGs) in pathological conditions [40-43]. Therefore, the inhibition of COX activity will prevent the conversion of AA to Prostaglandin H2 (PGH2), hence the inflammation prevention [44].

The prostaglandin E2 enzyme-linked immune-sorbent assay (PGE2 ELISA) is the most used test to detect COX inhibition as long as it is very sensitive, suitable for automation, and

ideal for the rapid screening of wide chemical agents [43]. The only drawback is that the assay does not discriminate between the activities of different COX isoforms [45].

This assay is based on the competition between PGs and a PG-acetyl cholinesterase conjugate for a limited amount of PG antiserum [44]. Figure 5 summarizes the protocol described by Walker and Gierse [41] and Chandrakanthan *et al.* [45].

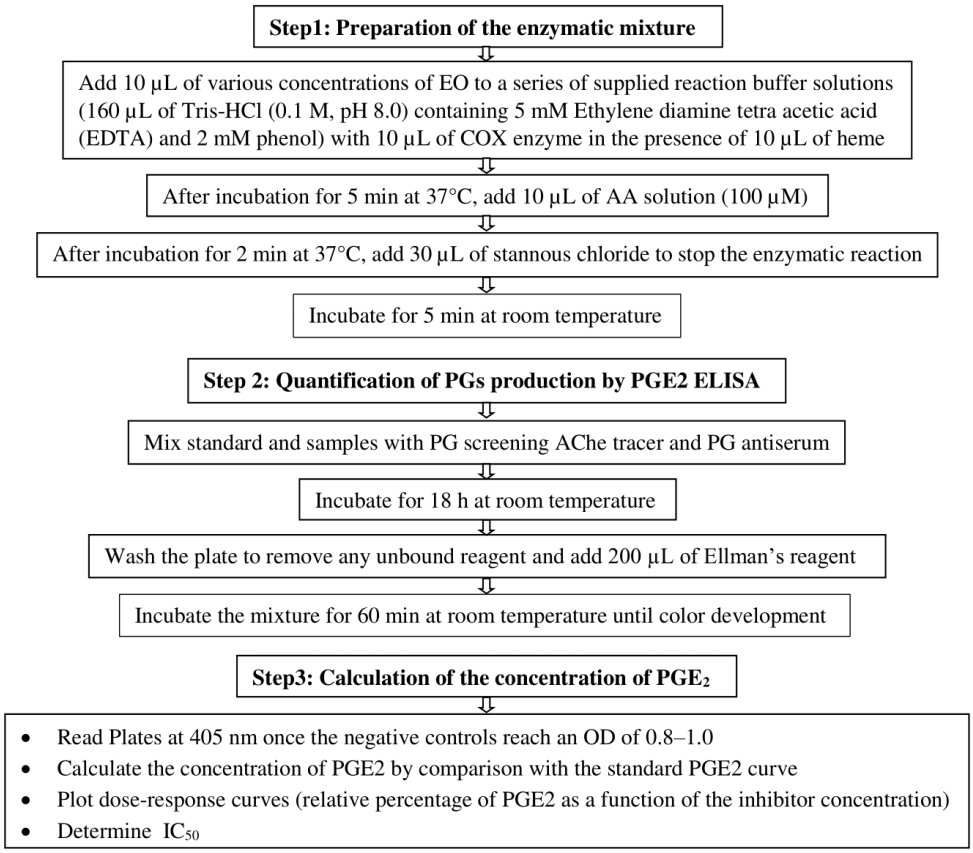

Figure 5. Experimental protocol of COX inhibition assay to assess the anti-inflammatory activity of EOs.

Note: The amount of PGE2 produced is expressed as a percentage relative to the positive and negative controls. The positive control contains DMSO without sample, and the negative control contains DMSO without AA [43].

3.3. Lipoxygenase (LOX) inhibition assay.

The LOX enzyme converts arachidonic acid or linoleic acid into various fatty acid metabolites involved in inflammation [47]. Therefore, the inhibition of LOX activity is important to prevent inflammatory diseases [48].

The spectrophotometric method is the most used to identify new LOX inhibitors since it is sensitive and rapid. Nevertheless, the reagents could rapidly oxidize, leading to an increasingly dark background. Also, the spectrophotometric readings must be precisely timed to quantify activity [47-49].

This assay is based on the enzymatic conversion of linoleic acid to conjugated dienes resulting in an increase in absorbance at 234 nm. The test protocol used by Njenga and Viljoen [47] is summarized in the diagram below (Figure 6).

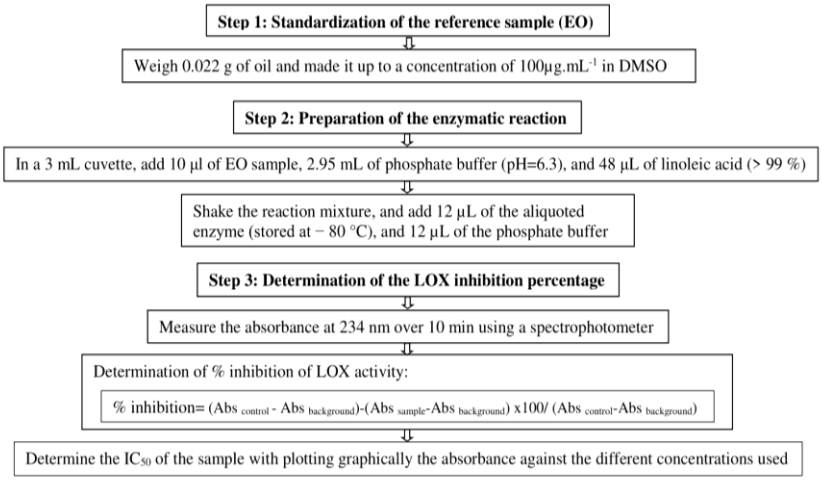

Figure 6. Experimental protocol of LOX inhibition assay to assess the anti-inflammatory activity of EOs.

***Note*:** Nordihydroguaiaretic acid (NDGA) can be used as a positive control, while the DMSO can serve as a negative control (no enzyme inhibition). The test is performed in triplicate [47,48].

4. Evaluation of anticancer activity

4.1. MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

The MTT assay is used to assess the *in vitro* cytotoxic and antiproliferative activity of EOs. It is a colorimetric assay that measures the formazan product (dark purple) formed through the reduction of MTT (yellow dye) by active cells [50]. A high absorbance rate indicates an increase in cell proliferation and vice versa [51].

Although the MTT assay is rapid and economical, it has some limitations, such as the evaporation of volatile solvents and the instability of the formazan's signal. The MTT assay protocol described by Russo *et al.* [51] is presented below (Figure 7).

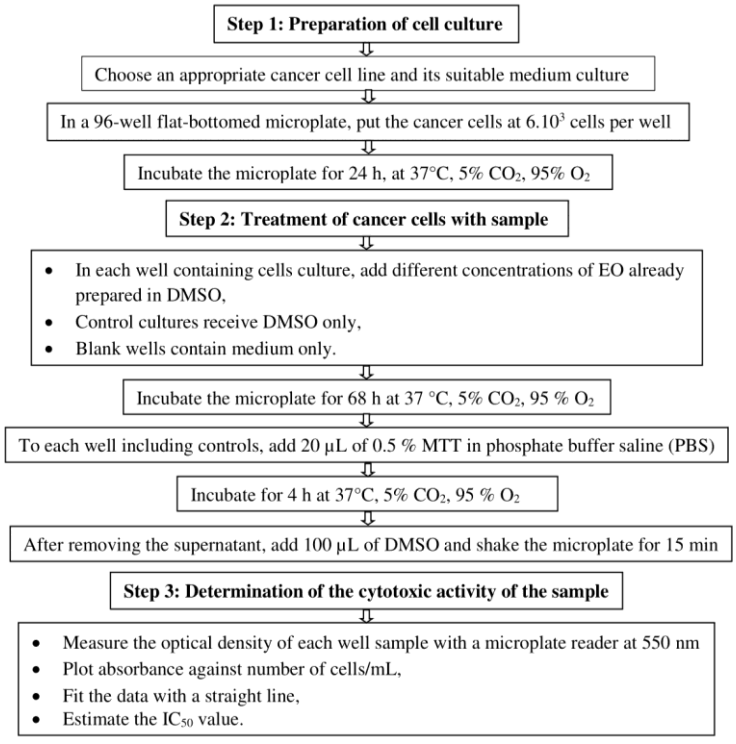

Figure 7. Experimental protocol of MTT assay for assessing EOs anticancer activity.

Note: The cytotoxicity is expressed as IC_{50}. Doxorubicin can be used as a positive control. The cell lines used in this protocol are from melanoma cancer: A375, M14, and A2085. Their respective suitable mediums are: DMEM supplemented with 10% fetal calf serum (FCS), 2.0 mM L-glutamine, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 25 µg/mL of fungizone; The Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 25 µg/mL fungizone, and DMEM supplemented with 10% FCS, 2.0 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM of nonessential amino acids. The experiment is conducted in triplicate [52].

4.2. Caspase-3 activity assay.

Caspase-3 is an intracellular cysteine protease. Its activation is known to trigger apoptosis [53]. As caspase-3activity prevents tumorigenesis [54], it is necessary to test the *in vitro* effect of natural products on caspase-3of cancer cells. This assay is based on p-nitroaniline (pNa) monitoring, which is released during the cleavage of a caspase-specific peptide by the caspase-3. pNA can be quantified at 405 nm [53,58]. This assay is a valuable, reliable, and time-saving technique for apoptotic studies. However, it is not quantitative; so, the resulting lysate cannot be used for downstream assays. Moreover, determining the appropriate timing to assay caspase activity can take some effort [55]. The diagram below (Figure 8) represents the protocol of caspase-3 activity assay described by Jamali *et al.* [57] and Szkoda [58].

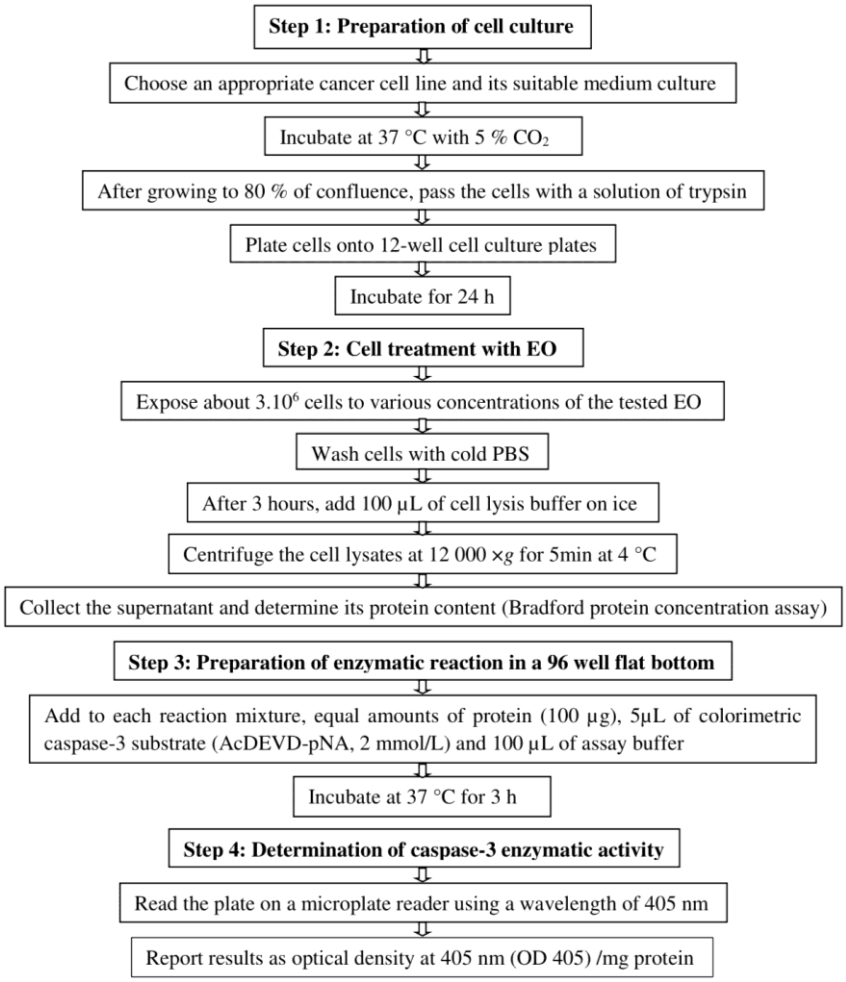

Figure 8. Experimental protocol of Caspase-3 activity assay to assess the anticancer activity of EOs.

Note: The color reaction is directly proportional to the level of caspase enzymatic activity. Thymol and doxorubicin can be used as a positive control. The dithiothreitol (DTT), or EDTA can be used as assay buffers as well as cell lysis buffers. Among cancer cell lines used in this essay are M624 melanoma cells and human mammary carcinoma cell lines; MDA-MB-231, MCF-7. Their suitable media are respectively : DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, and RPMI supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) [56-58].

5. Evaluation of antidiabetic activity

5.1. α-amylase and α-glucosidase inhibition assays.

α-amylase is a pancreatic enzyme that catalyzes the oligo and/or disaccharides into monosaccharides in the digestive system, while α-glucosidase is an intestinal enzyme that hydrolyzes complex carbohydrates as starch to mere glucose molecules, which cause an increase in postprandial blood glucose levels [59,60]. Hence, the α-amylase and α-glucosidase inhibition present a significant way to reduce blood glucose levels in the case of diabetes [61,62].

These assays are simple, reliable, and rapid; however, they require careful reagents preparation given their high sensitivity. Also, the turbidity resulting from enzymes' starch digestion may be a limitation of these tests [63]. Below, the test protocols of α-amylase inhibition assay (Figure 9) and α-glucosidase inhibition assay (Figure 10) described respectively by Bernfeld [64] and Oboh *et al.*[65].

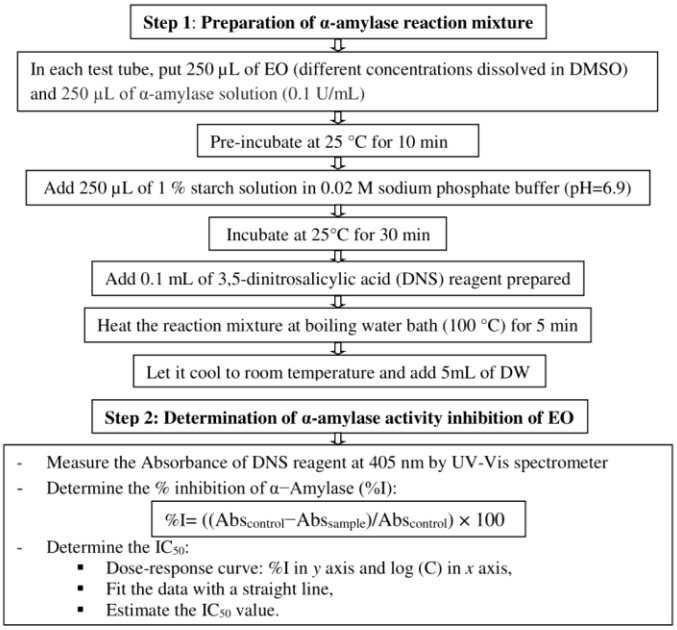

Figure 9. Experimental protocol of α-amylase inhibition assay to assess the antidiabetic activity of EOs.

***Note*:** α-amylase inhibition is expressed as a decrease in units of reducing sugar (maltose equivalent) in (µg/mL). DNS is prepared by mixing 12 g of sodium potassium tartrate tetrahydrate in 8 mL of 2 M sodium hydroxide (NaOH), and 96 mM DNS solution. The assay buffer is prepared by mixing 20 mM Sodium phosphate buffer with 6.7 mM Sodium chloride (NaCl), (pH=6.9). Negative control contains DMSO and assay buffer instead of EO. The positive one contains acarbose instead of EO. The assay is carried out in triplicate [64,66,67].

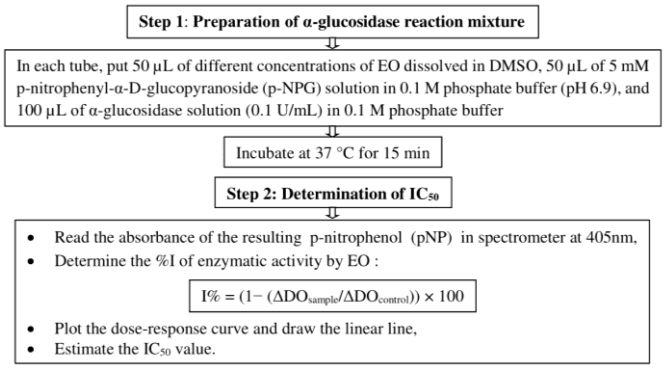

Figure 10. Experimental protocol of α-glucosidase inhibition assay to assess the antidiabetic activity of EOs.

Note: The absorbance of the resulting p-NP is directly proportional to the enzyme activity and the test was performed in triplicate [68]. Negative control contains DMSO, substrate, and enzyme. While positive control contains acarbose instead of EO [65,66].

6. Evaluation of antimicrobial activity

6.1. Broth dilution method (BDM).

The BDM is the most suitable *in vitro* test to determine antimicrobial agents' minimal inhibitory concentration (MIC). The MIC is the lowest concentration of an antimicrobial agent to inhibit the growth of a microorganism [69,70]. This method includes broth macro and micro dilutions [71].

The broth microdilution method is the most used since it is simple, economical, effective, and reproducible. Nevertheless, the method procedure must be carefully controlled to obtain reproducible results [72].

Figure 11 explains the experimental protocol of the broth microdilution method described by Balouiri *et al.* [73] and Nascente *et al.* [70] according to the clinical and laboratory standards institute (CLSI).

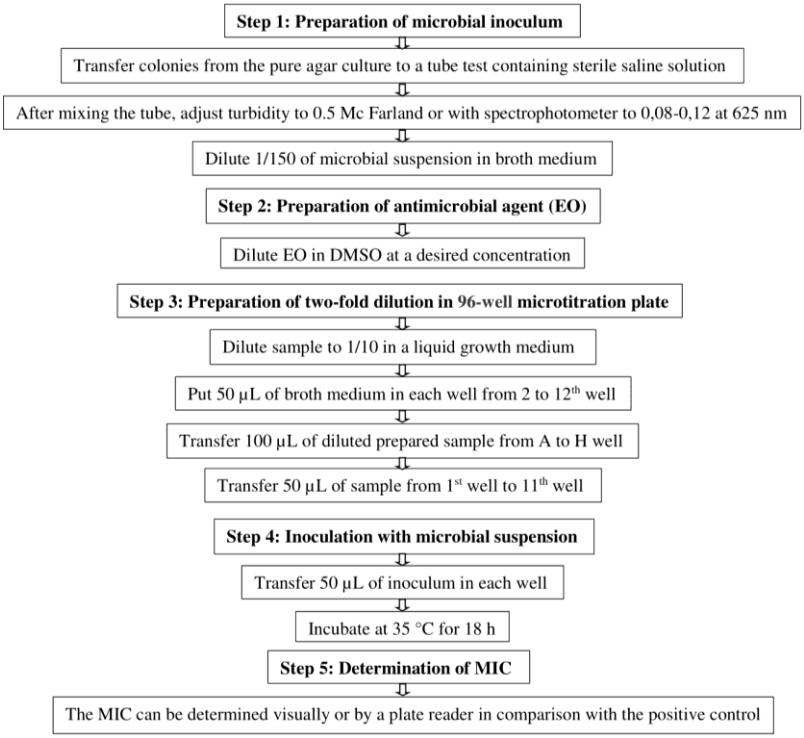

Figure 11. Experimental protocol of broth microdilution method to assess the antimicrobial activity of EOs.

Note: This test requires Mueller Hinton broth (MHB) as a culture medium for bacteria and Yeast Extract Peptone Glycerol (YPG) for yeasts. The final inoculum sizes required for bacteria and yeasts are 5.10^5 CFU/mL and (0.5–2.5) $\times 10^3$ CFU/mL, respectively. Column 12 is reserved as a positive control of growth. The MIC is expressed in µg/mL or mg/L. The BDM can also determine the minimum lethal concentration (MLC), defined as the lowest concentration killing 99.9% of the final microbial inoculum. The MLC is determined by sub-culturing the sample concentration (that does not show any microbial growth in wells) on the agar plate surface. Then, the number of surviving cells (CFU / mL) is counted after incubation for 24h [71-73].

6.2. Disc diffusion method (DDM).

DDM or Kirby-Bauer method tests the sensitivity of certain pathogenic bacteria and yeasts to the tested antimicrobial agents [81]. In this method, the antimicrobial agent diffuses from the filter paper disk to the agar surface, which contains the test microorganism. If there is an antimicrobial activity, an inhibition zone (IZ) will be developed around the disk after incubation [74,75].

The DDM is a simple, inexpensive, and standardized technique that tests enormous numbers of microorganisms and antimicrobial agents, with easy interpretation of the results provided. However, this method does not determine the MIC and cannot distinguish bactericidal and bacteriostatic effects. The recommended experimental protocol for the DDM (Figure 12), according to Singh *et al.* [76] is presented below.

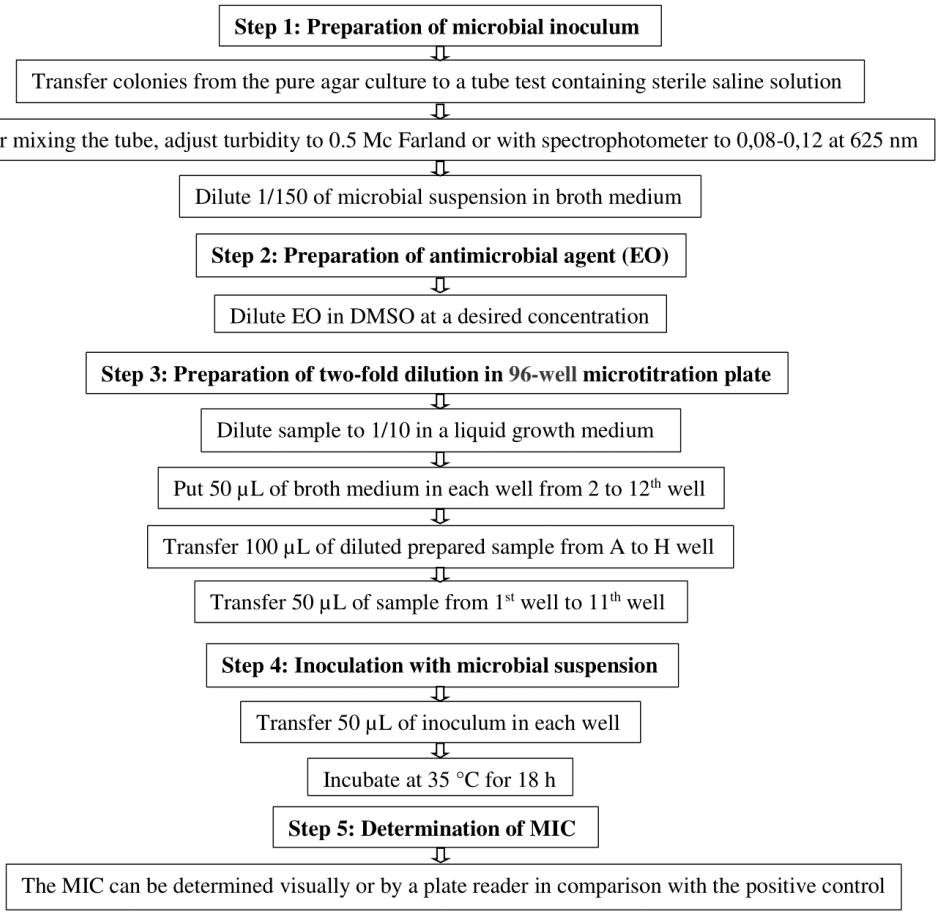

Figure 12. Experimental protocol of Disc Diffusion method to assess the antimicrobial activity of EOs.

Note: Some antibiotics can be used as reference controls for the bacteria tested, such as ampicillin, chloramphenicol, and streptomycin at the desired concentration per disk. The culture medium used for the yeasts is MHA supplemented with 2% of glucose and 0.5 mg/mL of methylene blue. Regarding the incubation conditions, the temperature used for bacteria and yeast is 35 °C, and the incubation time required for bacteria is 16-18h and for yeast is 20-24 h. The final inoculum size is adjusted to 0.5 McFarland, corresponding to (1–2) 10^8 CFU / mL for bacteria and (1–5)10^6 CFU / mL for yeasts [76-78].

7. Conclusions

The present review gathers the suitable *in vitro* methods to test antioxidant and some biological properties of EOs, particularly antidiabetic, antimicrobial, anticancer, and anti-inflammatory activities to highlight their therapeutic potential and create a guiding document for researchers working on EOs. Indeed, the *in vitro* methods indicated are simple, fast, reproducible, sensitive, inexpensive, and avoid animals use. However, they have some common limitations, particularly the demand of high concentrations to avoid errors, the monitoring of time interval, volatility ad solubility of the used solvents, the risk of contamination, and repeat experiments. Ultimately, studies showed that EOs are excellent sources of bioactive natural compounds with many pharmacological and biological properties beneficial for health and the environment. Therefore, studying the EOs bioactivities, action modes, and bioactive molecules is necessary to detect new therapeutic properties and new efficient agents for application in the medical and pharmaceutical field. Besides, EOs could represent a source of biopesticides and biodetergents, contributing to protecting the environment; hence the interest in the scientific evaluation of their biological properties via *in vitro* and *in vivo* methods.

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Conflicts of Interest

The authors declare no conflicts of interest.

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