Review Article

An Overview of Antimicrobial and Antioxidant Bioautography Method Analysis: C*osmos caudatus* and *Orthosiphon stamineus*

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Abstract

Medicinal herbs such as *Cosmos caudatus* and *Orthosiphon stamineus* are sources of a huge number of bioactive compounds with antioxidant and antimicrobial properties. *C. caudatus* plant has bioactive compounds such as phenolics, flavonoids, and terpenoids. On the other hand, phytochemicals from *O. stamineus* have been shown to have a wide range of traditional applications due to their antioxidant and antibacterial characteristics. The bioautography method is used to isolate active molecules on chromatogram followed by the chemical and biological detection system. In antimicrobial bioautography, agar overlay Thin Layer Chromatography (TLC) bioautography detection, direct TLC bioautography detection, and contact bioautography are used for antimicrobial screening of the compounds. Meanwhile, TLC 2, 2-diphenyl-1-picrylhydrazylradical (TLC-DPPH) bioautography is applied for the detection of antioxidant activity of the plant extracts. This review aims to list and describe the antimicrobial and antioxidant bioautographic methods used especially for *C. caudatus* and *O. stamineus* plants. This study might be useful in identifying the antimicrobial and antioxidant properties of the constituents of both plants.

Keywords: Antimicrobial, antioxidant, bioautographic, *Cosmos caudatus*, *Orthosiphom stamineus.*

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1.0 Introduction

Many analytical methods, including chromatographic techniques, have been used for separation and determination of flavonoids and phenolic acids and one of them is TLC bioautography (1). It is an effective and inexpensive technique for the phytochemical analysis of plant extracts. This can be performed in both highly equipped as well as in small research laboratories (2). TLC plays a significant role in analysing polyphenols in natural extracts and in verifying the existence of plant-based natural drugs. TLC bioautography is a method that integrates chromatographic separation with in situ assessment of biological activity (3). TLC bioautography can be used to conduct antibacterial, antioxidant, and enzyme inhibitory activities. This method is mainly used for preliminary screening of natural products possessing antimicrobial and antioxidant biological activities and for bioactivity-directed fractionation and isolation of active components from complex extracts. TLC-direct bioautography is also able to identify natural compounds that exhibit antimicrobial properties (2). In TLC DPPH, purple background, DPPH radical-scavenging chemicals emerged as yellow dots. In reverse phase TLC plates with DPPH as a detecting agent, the developing colour will be quite unstable, however, on standard TLC plates; the coloration formed after spraying with DPPH is relatively stable, allowing the identification of radical-scavenging activity after 30 minutes (4). *C. caudatus* and *O. stamineus* are said to have rich amounts of secondary active metabolites. *C. caudatus* contained mainly quercetin, quercitrin, rutin and chlorogenic acid while *O. stamineus* contained rosmarinic acid, sinensetin and many other compounds. In this review, the application of bioautography methods is discussed to identify the antioxidant and antimicrobial activity of plants phytoconstituents regarding *O. stamineus* and *C. caudatus*. By using this method, we can identify the phytochemicals present in both plants and evaluate the antioxidant and antimicrobial activity of the compounds. This method is more economical, versatile, consume less time, and easy to perform.

2.0 *Cosmos caudatus* species

C. caudatus is a herb of the family Asteraceae (5). It is locally known as Ulam Raja in Malaysia. It is a medicinal plant originating from Latin America and later grown in Southern Asia (6). The species is an annual to short-lived aromatic herb, grows to a height of 3 metres, and bears purple or pink coloured flowers (7). The leaves are pinnate and dissected into five leaflets in opposite directions. The leaf lamina's upper surface is dark green while the lower surface is bright green with minute hairs. The pinkish or violet daisy-like flowers are composited with a cluster of yellow florets at the centre. Due to its unique, appealing smell and aroma, it is eaten raw which adds diversity and taste to food (8). To enhance the flavours, it is usually eaten by dipping in shrimp and chili paste. *C. caudatus* leaf can be served in hotels and restaurants across Malaysia as a local delicacy. This plant has high antioxidant ability with specific medicinal properties, including anti-diabetic, anti-hypertensive, anti-inflammatory, bone-protection and antimicrobial activity (6).

Phytochemical properties

Studies conducted on *C. caudatus* have revealed a vast array of phytochemicals. Phenolic compounds of caffeoylquinic acids, quercetin glycosides, catechin, and proantho-cyanidins have been reported

from the leaves of *C. caudatus* (8). The caffeoylquinic acids are chlorogenic, neo-chlorogenic, cryptochlorogenic acids. Quercetin glycosides include arabino-furanoside, glucoside, rhamnoside, and rutinoside (9,10). Quercetin is the prevalent flavonoid in the *C. caudatus*, while chlorogenic acid, caffeic acid and ferulic acid are the major phenolic acids (11,12). The essential oil of *C. caudatus* contains γ-cadinene (33%) and caryophyllene (10%) as major components (13). The chlorogenic, neochlorogenic, crypto-chlorogenic acids isolation in *C. caudatus* were previously obtained by using aqueous acetone and aqueous ethanol fraction (8). Column and liquid chromatography were used for the isolation of quercetin and six of its glycosylated derivatives in *C. caudatus*. Quercetin is a heterocyclic compound , insoluble in water and was fractionated by ethyl acetate from the methanolic extract (10). Table 1 showed the main chemical constituents found in the leaves of *C. caudatus.*

Table 1: Phytochemicals identified in leaves of *C. caudatus* Kunth

Plant part	Compounds	Class	References
Leaves	Catechin	Flavonoids	(8)
	Quercetin 3-O-glucoside	Flavonoids	(8)
	Quercetin pentose	Flavonoids	(8)
	Quercetin deoxyl-hexose	Flavonoids	(8)
	Chlorogenic Acid	Phenolic Acids	(8,12)
	Neochlorogenic Acids	Phenolic Acids	(8)
	Crytochlorogenic Acid	Phenolic Acids	(8)
	Caffeic Acid	Phenolic Acids	(12)
	Ferulic Acid	Phenolic Acids	(12)

Table 2: Some important constituents present in aerial part of *O. stamineus* Benth

Plant part	Compounds	References
Aerial	α-humulene	(28)
	Eupatorin	(22)
	Eugenol	(28)
	Luteolin	(29)
	Oleanolic acid	(24)
	Quercetin	(29)
	Rosmarinic acid	(26)
	Sinensetin	(26)
	Salvigenin	(24)

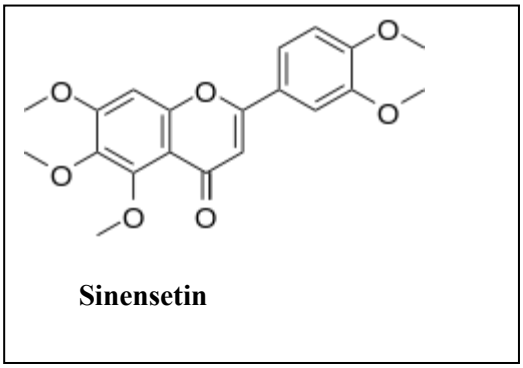
3.0 *Orthosiphon stamineus* species

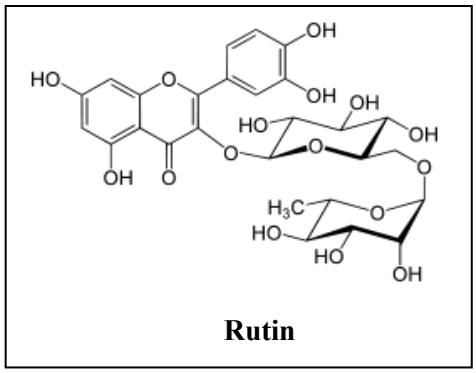
O. stamineus is widely grown in tropical areas. Tropical climate with high temperature and rainfall all year in Malaysian and Indonesia enabled the plant to flourish extensively (14). *O. stamineus* was introduced into Europe in the early 20th century, where it became a popular herbal health tea (15). Other names of this species are as *O. aristatus*, *O. spicatus*, *O. blaetter*, *Indischer Nierentee*, *Feuilles de Barbiflore*, and *de Java*. It is also commonly referred to as "Misai Kucing" in Malaysian or "Kumis Kucing" in Indonesian which means cat whiskers and it belongs to the Lamiaceae family (16). The purple and white varieties of *O. stamineus* exist in Malaysia and this could be differentiated merely based on the colour of corolla and calyx. It is graded according to the colour of the white and purple flowers (17). The purple type has yellowish spots that are scattered unevenly on the leaf surface and grow light purple flowers. This type contains more bioactive compounds compared to the white type (18). The white variety has green leaves on the surface of the leaves with white flowers, without yellowish spots. The herb is commonly called Java tea and is widely used in Asian herbal tea. Previous research stated that the leaves of the plant are commonly used as herbal tea for diuresis, to treat rheumatism, diabetes, urinary lithiasis, oedema, eruptive fever, influenza, hepatitis, jaundice, biliary lithiasis, and hypertension (14).

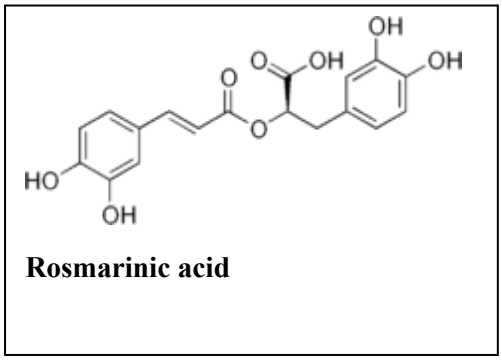
Phytochemical properties

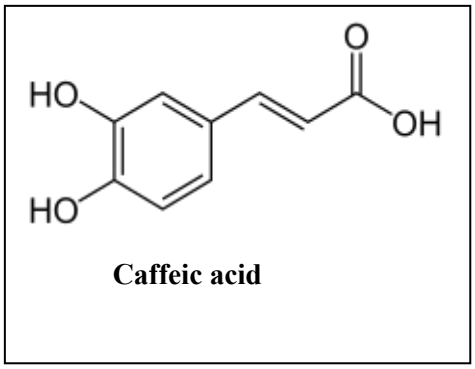
Chemical screening from this plant has revealed three types of phytochemicals in various extracts. The three phytochemicals are polymethoxylated flavonoids (19), phenylpropanoids (caffeic acid derivatives) (20) and terpenoids (diterpenes and triterpenes) (21). Isolation from the hydroalcoholic extract of *O. stamineus* leaves showed the most prominent flavonoids which are sinensetin, eupatorin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavones (22), tetramethyl-scutellarein (23), salvegenin, ladanein, vomifoliol, 7,3',4'-tri-O-methylluteolin, and scutellarein tetramethylether (24). Caffeic acid and its derivatives such as rosmarinic acid, cichoric acid, and 2,3-dicaffeoyl-tartaricacid are usually present in the aqueous extract of *O. stamineus* (25). In addition, terpenoids like orthosiphols A–Z, staminols A–D, orthosiphonones A–D, staminolactones A and B, secoorthosiphols A–C, norstaminols A–C siphonols A–E, and many other diterpenes were also detected (18). Studies have shown that seven triterpenes have been isolated from this plant ursolic acid, oleanolic acid, betulinic acid, hydroxybetulinic acid, maslinic acid, a-amyrin, and b-amyrin. Not only that, some essential oils were also detected, and they are mainly oxygenated monoterpene and sesquiterpene hydrocarbons. Over hundreds of chemical compounds have been documented as monoterpenes, diterpenes, triterpenes, saponins, flavonoids, organic acids. *O. stamineus* product contains poly-methoxylated flavonoids (sinensetin and eupatorin) and caffiec derivatives such as rosmarinic acid and many others (26). The enrichment of sinensetin in the leaves of *O. stamineus* were also quantified. Thin-layer chromatography, GC coupled with mass spectrometry (MS), UV, IR and ^{1}H-NMR spectroscopy were used (27). Previous research showed that *O. stamineus* contains many flavonoids when purified by TLC using methanolic extract (14).The main chemical constituents of *O. stamineus* are given in Table2. Some important phytochemicals reported in both plants are given in Figure 1.

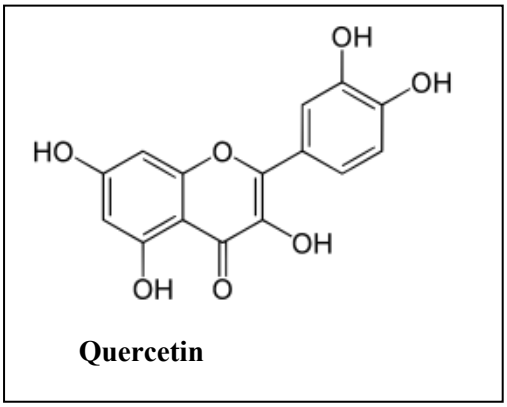
O. stamineus ***C. caudatus***











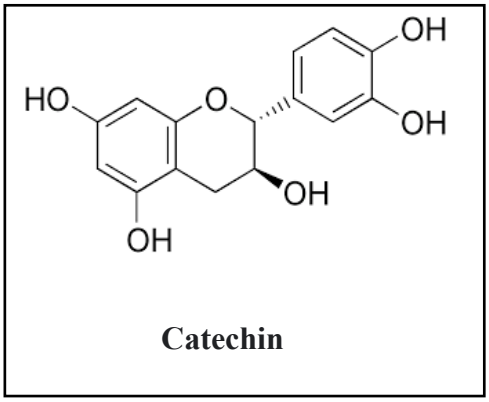


Figure 1: Important phytochemicals reported in *O. stamineus* and *C. caudatus*

4.0 Bioautography method analysis of extracts

Bioautography is the term used for planar chromatographic analysis hyphenated with biological detection method (30). It is a simple and cost-effective method to identify bioactive lead in plant extracts. It can be carried out in both highly established and simple laboratories (2). Despite the presence of sophisticated online high-performance liquid chromatography in combination with bioassays, bioautography provides a quick, easy and inexpensive approach for chemical and biological screening of complex plant extracts with subsequent bioassay-guided isolation (31). Bioautography's main applications are the rapid screening of a wide number of bioactivity samples including antibacterial, antifungal, antioxidant, enzyme inhibition and target-oriented isolation of active compounds (32–35). For detection of antimicrobial properties in a mixture of compounds, there are three methods of bioautography called immersion or agar overlay bioautography (TLC-IB), direct TLC bioautography (TLC-DB) and agar diffusion or contact bioautography (TLC-CB) (36,37) are used.

4.1 Antimicrobial Bioautography

Immersion bioautography is a combination of direct and contact bioautography. In this process, a molten is used to cover the chromatogram. The inhibition or growth ranges are visualized after solidification, incubation and staining (38). An agar solution containing the red coloured bacterium *Serracia marcescens* may be used for Gram-negative bacteria. At room temperature, the red-coloured gel is incubated overnight and zones of inhibition appear on a red background as white or light yellow areas (39). Some colourless microorganism zones of microbial growth inhibition can be visualized with the assistance of a dehydrogenase activity-detecting reagent called tetrazolium salt. Metabolically active microorganisms transform tetrazolium salt (MTT) into the resulting, brightly coloured formazan. Bacteria such as *B. subtilis, E. coli*, *Pseudomonas aeruginosa* and *S. aureus* can also be used in agar overlay assay (40,41).

In addition, the developed TLC plate is sprayed or dipped into a fungal or bacterial suspension in direct TLC bioautography. For spraying or dipping purposes, a suspension of test bacteria or fungi is applied. For bacteria such as *Staphylococcus aureus*, an absorbance inoculum of 0.84 at 560 nm is recommended (42), while a suspension of 10^6 CFU / mL can be used for both bacteria and fungi (43). In humid conditions, the chromatogram is then incubated at 25°C for 48 hours. Tetrazolium salts are used for the visualisation of microbial growth. These salts are transformed into brightly coloured formazan by the dehydrogenases of living microorganisms (44). Tetrazolium salts are sprayed onto the chromatogram and then let it to re-incubated for 24 hours at 25 °C (44) or 37 °C for 3–4 hours (45,46). Clear white areas on the TLC plate shows antimicrobial activity of the sample against a purple background (47).

In contact bioautography, antimicrobial agents diffuse from a developed TLC plate or paper to an inoculated agar plate (41,48,49). For a specific period, the chromatogram is placed face down onto the inoculated agar layer to allow for diffusion. After that, the chromatogram is removed, and the layer of agar is incubated. The antimicrobial compounds are indicative of inhibition zones on the agar surface, comparable to the spots in chromatographic plates. Figure 2 showed different antimicrobial bioautographic

methods. An experiment was carried out to analyse crude n-hexane, diethyl ether (Et_2O), ethanol (EtOh) and phosphate buffered saline (PBS) extracts of *C. caudatus* leaves for their antimicrobial action against five microbial strains using disc diffusion method. There were two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), two Gram-negative bacteria (*Escerichia coli* and *Pseudomonas aeruginosa*) and one fungal (*Candida albicans*). The inhibitions of n-hexane, Et_2O and EtOh extracts were observed against all the microbes tested in this experiment, except for PBS extract (50). *C. caudatus* ethanolic extract was also investigated against ten strains of food-pathogenic bacteria by disc diffusion method. Results showed that ethanolic extract was found to be active against several strains of pathogenic human bacteria such as S*almonella sp*, *Proteus* mirabilis, *Salmonella typhimurium Listeria monocytogenes, Staphylococcus aureus* and *Vibrio cholera* (51). Moreover, methanolic extract of *C. caudatus* was also tested against numerous foodborne for antibacterial pathogens, including *Bacillus cereus*, *Bacillus subtilus, Proteus mirabilis, Pseudomonas aeruginosa*, and *Candida albicans*. bacterial species had a shown broader inhibitory zone than fungal species across all pathogens studied. This demonstrates that fungi were more resistant to *C. caudatus* extract. *Pseudomonas aeruginosa* and *Proteus mirabilis* showed smaller inhibitory zones than *Bacillus cereus* and *Bacillus subtilis* (52).

An experiment explored the antimicrobial effects of *O. stamineus* extract and has been tested against selected foodborne bacteria *in vitro* using a disc diffusion assay (53). The 50% methanol extract of *O. stamineus* demonstrated variable antibacterial activity against various microbes with the strongest growth inhibitory action against *V. parahaemolyticus*. This is a bacterium that causes moderate human gastroenteritis following consumption of contaminated seafood. Analysis for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) verified the successful inhibition of *V. parahaemolyticus* development by *O. stamineus* methanol extract. This is possibly attributable to the high concentration of rosmarinic acid contained in the extract of *O. stamineus* that appeared to have significant antibacterial and free radical scavenging activities (53).

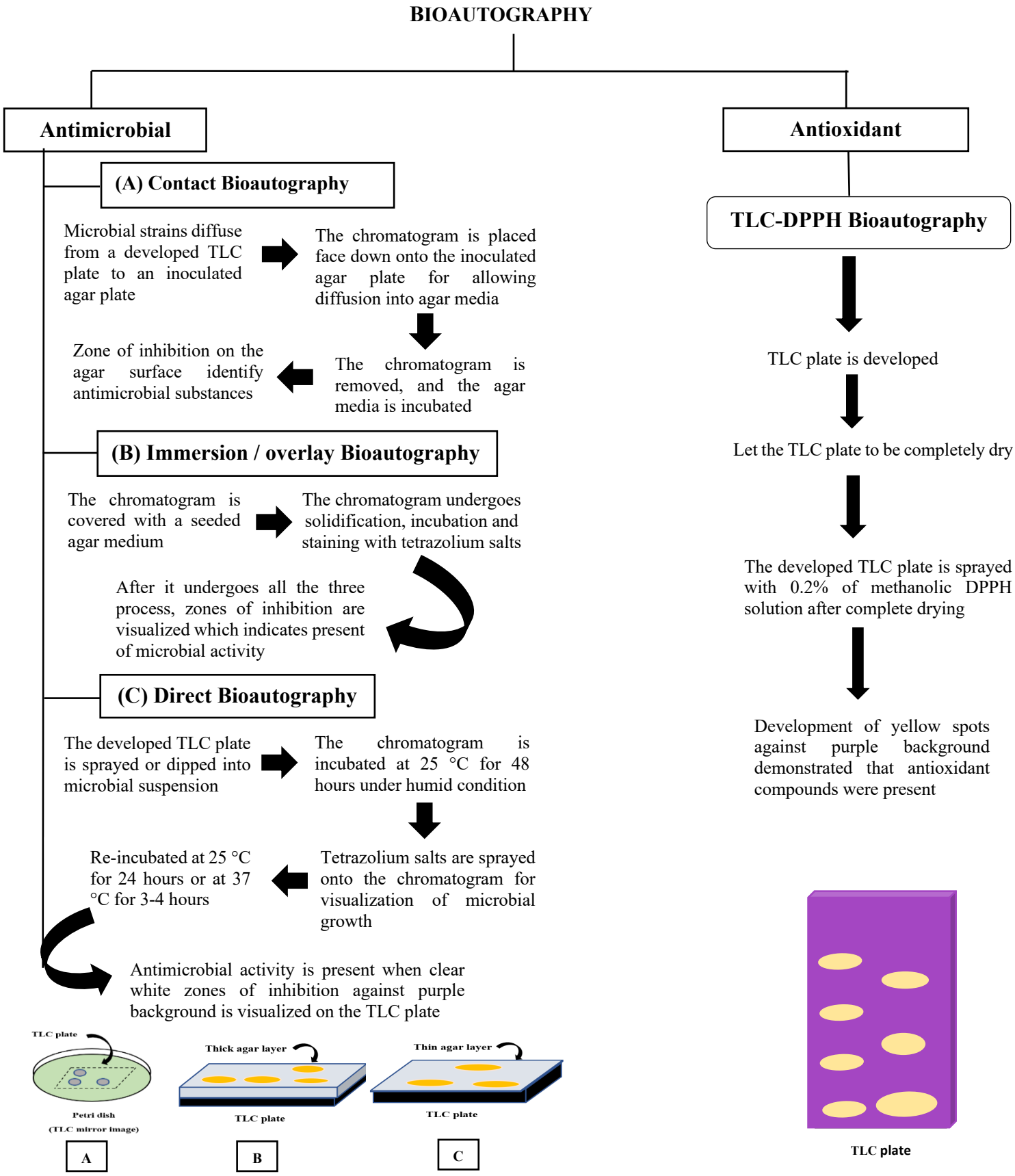


Figure 2. Flow chart shows different antimicrobial and antioxidant bioautographic methods

4.2 TLC- DPPH Bioautography

TLC-DPPH bioautography is used for the identification of antioxidant properties. The stable 2, 2-diphenyl-1-picrylhydrazylradical (DPPH) has a maximum absorption at 517nm which decreases after reduction by reaction with a radical scavenger and the resulting change of colour can be detected in a TLC bioassay. Thus, DPPH assay is commonly used to determine the properties of plant constituents for free radical scavenging. DPPH is a free radical that produces a violet solution in methanol. Antioxidants in the sample react to DPPH-free radicals and break the chain reaction and further free radicals formation. This causes a change of colour from purple to yellow. In this process, well-separated bands on purple background that exhibit yellow spots formation are considered to have antioxidant potential. The intensity of the yellow colour can be measured with a chromameter. Therefore, in pharmaceutical research, TLC has a broad application since it is less time consuming, low cost, and can be done with less complicated technique (54). Figure 2 showed DPPH bioautographic method.

A study reported the antioxidant potential and phenolic content using leaves extract of five plants including *C. caudatus.* Decolouration of the stable free DPPH radical by antioxidants in samples were measured by spectrophotometer. *C. caudatus* extract showed maximum free radical scavenging potential (86.85%) (55). In another study, the free radical scavenging activity of methanolic extracts of twenty one tropical plants via DPPH assay of *C. caudatus* extract showed IC_{50} value 21.3 µg/ml (56). A study also evaluated the antioxidant of *C. caudatus* leaves at different maturity stages (young, mature, and old leaves). The IC_{50} values of DPPH scavenging activity are increased as the age of *C. caudatus* leaves grew, demonstrating that young leaves exhibited more antioxidant activity than mature and old leaves.

Che Mansor et al., have found that the antioxidant capacity in *O. stamineus* is influenced by the extraction method (57). Reflux, Soxhlet and maceration methods were employed in this experiment. Results showed that highest total extraction yield was obtained from reflux (72.73%) followed by Soxhlet (62.51%) and maceration (37.78%). In addition, an *in vitro* model of DPPH scavenging was used to explore the antioxidative potency of different fractions of *O. stamineus* extract. All the extracts, including sinensetin (SEN), eupatorin (EUP), 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (TMF), and rosmarinic acid (RA), were shown to have antioxidant potencies equivalent to other common antioxidants, such as quercetin and butylated hydroxyanisole (BHA)(58).

5.0 Conclusion

The bioautography technique provides a time-efficient, adaptable, and cost-effective approach for chemical and biological screening of complex plant extracts with subsequent bioassay-guided isolation. Bioautography is particularly important to avoid the time-consuming isolation of inactive compounds. TLC bioautographic approaches incorporate chromatographic separation and determination of in situ activity to enable the localization and target-directed isolation of active constituents. With many advantages of these techniques, there are also some limitations of this study; the separation process is not simple for natural substances, quantity isolated if too small, will be difficult to detect biological activity. Considering these limitations, it is necessary to create a technique that can detect lead in tiny quantities while simultaneously measuring biological activity successively. In addition to that,

although it is a very fast and economic technique to analyze antimicrobial and antioxidant constituents, very limited data is available on *C. caudatus* and *O. stamineus*. Finally, it is recommended that these methods might be helpful in screening the phytoconstituents of these plants as a future study for the researcher.

Conflict of interest: Declared none

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