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Research Article

EVALUATION OF ANTIBACTERIAL, ANTIFUNGAL AND ANTIOXIDANT POTENTIAL OF ESSENTIAL OIL FROM *AMYRIS BALSAMIFERA* AGAINST MULTI DRUG RESISTANT CLINICAL ISOLATES

PRAVEEN DAHIYA[1*], AKSHI MANGLIK[1]

Amity Institute of Biotechnology, Amity University- Noida, Uttar Pradesh, India

Email: pdahiya@amity.edu/praveen_sang@yahoo.com

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ABSTRACT

Objective: To investigate the phytochemical constituents, TLC bioautography and antioxidants of *Amyris balsamifera* essential oil. The antimicrobial potential was also determined against various multi drug resistant clinical isolates.

Methods: Preliminary phytochemical analysis was performed. The antimicrobial potential of essential oil from Amyris was evaluated by agar well diffusion method against multi drug resistant clinical isolates. The antibacterial effect was investigated using the TLC-bioautographic method. The antioxidants analyzed include catalase, peroxidase, superoxide dismutase, glutathione-S-transferase and glutathione reductase.

Results: Phytoconstituents analysis demonstrated the presence of few phytochemicals present including saponins, terpenoids and phlobatanins. *Amyris balsamifera* essential oil was further investigated for its antimicrobial activity against twelve Multi drug resistant pathogenic bacteria and three fungi respectively. The oil showed broad antimicrobial activity against MDR Gram-positive bacteria and Gram-negative bacteria and fungal isolates such as *Staphylococcus aureus, Salmonella paratyphi, Escherichia coli, Klebsiella pneumoniae and Candida albicans.* The highest *in vitro* inhibitory activity was observed for *Klebsiella pneumoniae* with wide inhibition zone diameters (20±0.11 mm) followed by *Staphylococcus aureus* (18±0.15) mm. Among fungal isolates, the growth of only *Candida albicans* was inhibited. Thin layer chromatography bioautography assay demonstrated one large growth inhibition zone observed at *Rf* values of 0.63 against *Klebsiella pneumoniae* and *Staphylococcus aureus* 1. *Amyris balsamifera* essential oil was found to be rich in antioxidants such as superoxide dismutase, glutathione-S-transferase and glutathione reductase.

Conclusions: It can be concluded that, Amyris essential oil with good antimicrobial activity against several multi drug resistant clinical isolates and possessing antioxidant activity, thus can be used in the treatment of various microbial infections.

Keywords: Antibacterial activity; Antifungal activity; Antioxidant potential; *Amyris balsamifera*; TLC bioautography

INTRODUCTION

The spread of antibiotic-resistant strains of bacteria is one of the most serious threats to successful treatment of microbial diseases as it may render the current antimicrobial agents insufficient to control the diseases. The continuous emergence of multi drug resistant organisms poses serious threat to the treatment of infectious diseases. Down the ages essential oils and other extracts of plants have evoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases [1]. World Health Organization (WHO) noted that majority of the world's population depends on traditional Medicine for primary healthcare. Various infectious diseases have been known to be treated with herbal remedies for the betterment of mankind throughout the globe. Thus, scientists are increasingly turning their attention to natural products, either as pure compounds or as standardized plant extracts, looking for new leads to develop better drugs against microbial infections [2].

Essential oils (also called volatile oils) are the concentrated, hydrophobic liquids containing volatile aromatic compounds from plants. They are naturally synthesized by plants for different reasons according to their needs. They are called "*essential*" - because they carry the very essence, and undoubtedly the most important part of the plant. They can be obtained by expression, fermentation or extraction but the method of steam distillation is most commonly used for commercial production. Essential oils are a rich source of biologically active compounds [3]. Their potential antimicrobial traits are due to compounds synthesized in the secondary metabolism of plants.

Amyris balsamifera known as torchwood belongs to the family Rutaceae. *A. balsamifera* is a small evergreen tree grows in the Caribbean area and along the Gulf of Mexico. It is also found in tropical Asia especially India, Sri Lanka, Malaysia, Indonesia and Taiwan. Amyris oil or West Indian sandal wood oil is obtained by steam distillation of the wood from this tree which is pleasantly woody with a balsamic touch. It is much cheaper than sandalwood oil and is used as a fixative in perfumes. Amyris oil is rich in sesquiterpene alcohols, six sesquiterpenes were isolated and identified to be 10-epi-γ-eudesmol, α-agarofuran, 4-hydroxydihydroagarofuran, valerianol, β-eudesmol and elemol [4]. Sandalwood oil has been used to treat skin eruptions and inflammatory diseases in India for centuries. Amyris oil has historically been associated with antiseptics, wound cleaners, childbirth recovery, diarrhea and influenza. It is also reportedly used in Chinese medicine for the relief of stomach ache, vomiting and gonorrhoea. In Europe it was also used for the treatment of pains, fevers and 'strengthening the heart' [5].

The present study aimed at evaluating the phytochemical screening, *in vitro* antimicrobial activity and antioxidant potentials of *Amyris balsamifera* essential oil against multi drug resistant clinical isolates.

MATERIALS AND METHODS

Acquisition of *Amyris balsamifera* essential oil

Commercial brands of *Amyris balsamifera* oil (Amyris) was purchased from Delhi, India. As per manufacturer's information, it was prepared by steam distillation. The oil was further distilled by rotary evaporator. The essential oil was dissolved in methanol (0.3 ml oil/ 2 ml methanol). The oil was transferred into sterile vials and stored at 4°C till further analysis.

Microbial cultures and Growth conditions

The microbial cultures included multi-drug resistant isolates of *Enterobacter* sp, *Salmonella paratyphi*, *Salmonella typhi*, *Staphylococcus aureus, Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter* sp. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and sub-cultured on to nutrient broth for 24 h prior to testing. Three fungal isolates studied includes *Candida albicans, Aspergillus niger* and *Rhizopus nigricans.* The cultures were

maintained on potato dextrose agar at 4°C. These microbial isolates served as test pathogens for antimicrobial activity assay.

Phytochemical analysis

The oil dissolved in methanol (0.3 ml oil/ 2 ml methanol) was subjected to phytochemical screening for the presence of saponins, tannins, steroids, phlobatanins, anthraquinones, cardiac glycosides, alkaloids, reducing sugars and flavonoids by using wet reactions [6-7].

Antimicrobial activity assay

The agar well diffusion method was employed with slight modifications to determine the antibacterial activities of Amyris oil in methanol [8]. About 25 ml of nutrient agar and potato dextrose agar was poured into each petri plate. Once the agar solidified, the cultures were inoculated on the surface of the plates (1×10^8 cfu/ml). Subsequently, the surface of the agar was punched with a 6 mm diameter wells. Each well was filled with 50 µl of oil in methanol. The concentration of the extracts employed was 20 mg/ml. Control wells containing the same volume of methanol were made. After 24 h incubation at respective temperatures, the plates were observed for zones of growth inhibition, and the diameter of these zones was measured in millimeters. All tests were performed in triplicate and the antimicrobial activity was expressed as the mean of inhibition.

TLC bioautography assay

Amyris essential oil exhibiting significant antimicrobial potential against *Klebsiella pneumoniae* and *Staphylococcus aureus* 1 as determined by agar well diffusion method (Table 2) was analyzed using TLC bioautography assay. About 10 µl of oil in methanol was applied on glass coated silica gel plates. The plates were developed with toluene and ethyl acetate (93:7 v/v). The TLC plates were run in duplicate. One of the strips was visualized under UV light to see if the separated spots were UV active after which it was sprayed with 2% vanillin sulphuric acid reagent, the second strip was used for bioautography assay. Individual *Rf* for each spot was measured. TLC bioautography was carried out using the selected strains of bacteria. The developed TLC plates were thinly overlaid with molten nutrient agar inoculated with an overnight culture of bacteria. The plates were incubated in a dark and humid chamber overnight at 37°C. Subsequently, the bioautogram was sprayed with an aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride and further incubated for at 37°C for 4 h. Microbial growth inhibition appeared as clear zones against a pink background. The *Rf* values of the spots showing inhibition were determined.

Antioxidant activity of essential oil

Amyris oil was evaluated for the presence of various antioxidants including superoxide dismutase (SOD), catalase (CAT), glutathione-s- transferase (GST), reduced glutathione (GSH) and lipid peroxidation. Superoxide dismutase activity was measured by the NBT reduction [9]. Catalase activity was estimated by measuring the rate of decomposition of hydrogen peroxide at 240nm [10]. Assay of glutathione-s- transferase was done according to Habig et al. [11]. Lipid peroxidation was measured by using the TBA method as per Ohkawa et al. [12]. Glutathione activity (GSH) was assayed based on the reaction with DTNB [13]. Data was expressed as mean± standard deviation (SD).

RESULTS

Preliminary phytochemical analysis of *Amyris essential* oil demonstrated the presence of few phtyochemicals including, saponins, terpenoids and phlobatanins. Most of the phytochemicals tested including tannins, glycosides, steroids, anthraquinones and reducing sugars were not observed in Amyris oil (Table 1).

Table 1: Phytochemical analysis of *Amyris balsamifera* oil

Phytoconstituens	Amyris oil
Tannins	-
Saponins	+
Reducing sugars	-
Steroids	-
Glycosides	-
Terpenoids	+
Phlobatanins	+
Flavonoids	-
Anthraquinone	-

a) +: Positive, b) −: Negative

Table 2: Antibacterial and antifungal activity of Amyris essential oil determined by agar well diffusion assay

Test Microorganism	Zone of Inhibition (in mm)
Bacterial Isolates	
Acinetobacter sp.	13±0.22
Escherichia coli 1	9±0.10
Escherichia coli 2	9.6±0.12
Enterobacter aerogenes	-
Klebsiella pneumonia	20±0.11
Salmonella typhi	-
Salmonella paratyphi	11±0.09
Staphylococcus aureus 1	18±0.15
Staphylococcus aureus 2	9.2±0.085
Pseudomonas aeruginosa	-
Fungal Isolates	
Candida albicans	10.8±0.12
Aspergillus niger	-
Rhizopus nigricans	-

Zone of inhibition is expressed as mean± standard deviation, -: no inhibition

Amyris balsamifera essential oil was further investigated for its antimicrobial activity against twelve Multi drug resistant pathogenic bacteria and three fungi respectively (Table 2). The oil showed broad antimicrobial activity against MDR Gram-positive bacteria and Gram-negative bacteria and fungal isolates such as *Staphylococcus aureus, Salmonella paratyphi, Escherichia coli, Klebsiella pneumoniae and Candida albicans.* The highest *in vitro* inhibitory activity was observed for *Klebsiella pneumoniae* with wide inhibition zone diameters (20±0.11 mm) followed by *S. aureus* 1 (18±0.15) mm and Acinetobacter sp (13±0.22). The oil showed poor antifungal activity and inhibited the growth of only *Candida albicans*. No inhibitory activity was observed against the fungal isolates *Aspergillus niger* and *Rhizopus nigricans*.

TLC Bioautographic assay are usually used to screen the antimicrobial activity by separating components onto the surface of chromatographic plates and overlaying the TLC plate with molten bacterial agar. TLC analysis revealed the presence of saponins in the essential oil tested (data not shown). TLC bioautography was performed for Amyris essential oil against *Klebsiella pneumoniae* and *Staphylococcus aureus* 1 isolates. One large inhibitory zone with *Rf* value 0.63 was observed against the growth of isolates *Klebsiella pneumoniae* and *Staphylococcus aureus* 1 on the TLC plates B and C as white spot on pink background when sprayed with aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride (Figure 1).

Antioxidants namely SOD, CAT, GSH, GST and lipid peroxidation in *Amyris balsamifera* oil were analyzed as reported in Table 3. Amyris was found to possess high amount of GSH (19.89±0.091µg/mg) and GST (14.2±0.132µg/mg) followed by SOD (10.2±0.035 U/mg). Small amount of CAT and lipid peroxidation was observed.

Table 3: Level of enzymatic and non-enzymatic antioxidants in *Amyris balsamifera* essential oil

Species	Reduced Glutathione (µg/mg)	Glutathione -S-transferase (µg/mg)	Catalase (µg/mg)	Super oxide dismutase (U/mg)	Lipid peroxide assay (µg/mg)
Amyris balsamifera	19.89±0.091	14.2±0.132	2.62±0.072	10.2±0.035	4.43±0.163

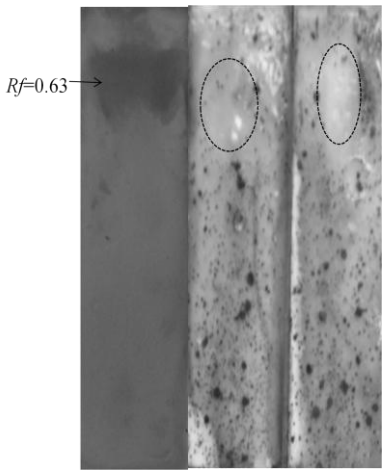

Plate A) (Plate B) (Plate C)

Fig. 1: Chromatogram for (Plate A) and Bioautograms (Plates B and C) for Amyris essential oil against *Klebsiella pneumoniae* and *Staphylococcus aureus* 1

Plate A: arrow indicates spot visualized when sprayed with 2% vanillin sulphuric acid reagent. Zones of inhibition (Plates B and C) are observed as clear spots against pink background. Mobile phase: Toluene/Ethyl acetate (93:7 v/v)

DISCUSSION

Plant essential oils have immense potential to be used as antimicrobial compounds. Thus, they can be used in the treatment of infectious diseases caused by the numerous resistant microorganisms present. Technically, essential oils are not true oils as they contain no lipid content. The essential oil is made up of a variety of complex, volatile compounds so they are a rich source of biologically active compounds. Therefore, it is reasonable to expect a variety of plant compounds in these oils with specific as well as general antimicrobial activity and antibiotic potential [14]. An estimated 3000 essential oils are known, of which 300 are commercially important in fragrance market [15]. These are also used in food preservative, aromatherapy, as muscle relaxant and as stimulant. Essential oils have been shown to possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties [16-17]. Some oils have been used in cancer treatment [18]. In the present study, medicinally important *Amyris balsamifera* essential oils was screened for the presence of phytoconstituents, antimicrobial potential, separation and analysis of bioactive compounds by TLC bioautography and anti oxidant enzyme analysis.

Phytoconstituents anaysis of Amyris oil showed that the oil contains few of the phytoconstituents including saponins, terpenoids and phlobatanins. Beek et al. [19] reported that the oil consisted of 17.5% sesquiterpene hydrocarbons and 82.5% oxygenated sesquiterpenes. Terpenoids encompass a diversity of structures and have many functional roles in nature, including protection against pest arthropods. Naturally occurring sesquiterpenes contained in Amyris oils are significantly repellent to a spectrum of arthropod pests and ticks [20-21].

In vitro antimicrobial activity by agar well diffusion method of Amyris essential oil was quantitatively assessed on the basis of zone of inhibition. Amyris oil in methanol exhibited varying degree of inhibitory effect against the selected multi drug resistant bacterial and fungal clinical isolates. The results are consistent with the reports of previous investigators. The antimicrobial activity of Amyris oil against the yeast *Candida albicans*, the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacteria *Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumonia has* also been reported by Jirovetz et al. [22]. Kloucek et al. [23] and Setzer et al. [24] reported antibacterial activity against of Amyris oil against *S. aureus*.

TLC was carried out in order to separate the bioactive components present. TLC is an easy and cost-efficient technique used in the separation of components of complex mixtures, commonly used for natural products. The main benefits of this technique include low cost analysis, high-throughput screening of samples, and minimal sample preparation [25]. An additional benefit is that the chromatograms can be screened for antimicrobial activity. The bioactive components were separated on TLC followed by TLC bioautography of Amyris essential oil against clinical isolates *K. Pneumoniae* and *Staphylococcus aureus* 1. One large inhibitory zone with *Rf* value 0.63 was observed against the growth of both *K. Pneumoniae* and *Staphylococcus aureus* 1 on the TLC plates B and C as white spot on pink background when sprayed with aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride. It is possible that the observed inhibition was likely due to one or more active compounds which overlap possibly due to the solvent system used for screening. Synergism might play a major role in extracts that were active when the MIC of the mixture was determined, while the separated components showed no antimicrobial activity.

Amyris balsamifera oil was analyzed for the presence of various antioxidants. Amyris was found to possess high level of GST, GSH followed by SOD and small amount of CAT and lipid peroxidation. Catalase is regarded as one of the most significant antioxidant enzyme that can protect plants by scavenging free radicals and H_2O_2. Low level of catalase was observed in *Amyris balsamifera w*hereas significant amount of SOD was recorded. SOD prevents the formation of .OH and provides essential defence against the potential toxicity of oxygen. High level of GST and GSH are observed in Amyris essential oil. GST offers protection against LPO by the conjugation of toxic effect with GSH [26].

CONCLUSIONS

The results of the present study support partially the use of the selected essential oil in traditional medicine notably in the treatment of microbial infections. A good antimicrobial compound coupled with high antioxidant activity is a very interesting lead compound to fight with the present scenario of multidrug resistance. It would have a dual role to inhibit the growth of MRSA and reduce the inflammation triggered by this pathogen by its high antioxidant activity. However further elucidation of the chemical structures of the biologically active compounds will be required along with studies in animal model to generate a potent drug.

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Conflict of interest statement

We declare that we have no conflict of interest.

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