PLANT SCIENCE RESEARCH AND PRACTICES

The Essential Guide to Plant Oils

Bente M. Holst
Editor

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PREFACE

This collection opens with a review of the plant sources, extraction methods, physicochemical characterization and commercial applications of edible and non-edible vegetable oils.

The authors describe the extraction of essential oil through ultrasound to increase the productivity of favorable essential oils. The operation of ultrasound-assisted extraction along is also described.

In one study, the antimicrobial activity of the essential oil derived from Campomanesia aurea O. Berg leaves is evaluated, and its antibiotic modulating potential is assessed.

In a subsequent study, the authors investigate the chemical composition of compounds from E. intermedium leaf essential oil, in addition to its modulatory and antibacterial action.

Additionally, the biological aspects attributed to the medicinal benefits of sandalwood oil and alpha-santalol are presented along with relevant clinical evidence.

Chapter 1 - Vegetable oils are obtained from oilseeds, fruits, or nuts by different pressing methods, solvent extraction or a combination of these methods. The main components of vegetable oils are saturated and unsaturated fatty acids. The yields, different compositions, and physicochemical properties determine the oil usefulness in various applications aside edible uses, such as in foods, pharmaceuticals, cosmetics

and production of energy. Economic development and populational growth have boosted the demand for vegetable oils. The chapter includes plant sources, extraction methods, physicochemical characterization and commercial application of edible and non-edible vegetable oils. Patent documents about vegetal oils recorded in Espacenet and WIPO Patentscope databases, as well as the countries, applicants and International Patent Classification (IPC) with the main records and relative percentage in WIPO Patentscope are presented. At the end of the chapter, future outlooks on the subject are provided.

Chapter 2 - Minimal or negligible side effects of secondary metabolites obtained from plant material explain the growth of the essential oil (EO) industry. Essential oils are the naturally occurring metabolites known as valuable chemicals that possess several beneficial characteristics and can replace the chief synthetic supplements. EOs are widely recognized for their aromatic, medicinal, acaricidal and antibacterial properties. These characteristic properties have taken over a variety of synthetic medicines as natural products defined as ‘generally recognized as safe products’. A variety of EOs as java citronella oil, mint oil, patchouli oil, etc. with varying composition of valuable chemicals has found its place in the industrial market of flavor, fragrance, cosmetic and medical field. This drive has increased the demand for EO sequentially in the market. The study has proven the upsurge of the natural product based cosmetic market in the United States by 9% and the United Kingdom by 8%. The commodity export from India was increased by 37%. The hydrophobic valuable compounds obtained from the plant material are volatile and can be easily extracted from various parts of plant material. However, because of their lower availability in plant material, it is important to extract them effectively by developing an enhanced technique. To avail their magnificent characteristics ultrasound-assisted extraction could be a promising option. Many researchers have extracted the essential oil using ultrasound wherein physical damage caused by cavitation bubbles leads to an increase in the productivity of essential oil. This chapter would describe the extraction of essential oil through ultrasound to increase the productivity of favorable essential oils. Later, the operation of ultrasound-assisted extraction along with mechanism will be

described. A variation in sonication parameters may affect the extraction efficiency which is discussed in detail in this chapter. Also, the comparison of various techniques is made to show its environmental legibility, improved extraction in terms of extraction time, quantity and quality of natural product. Later, various optimization techniques utilized for extraction will be discussed. Also, the upscaling of the method will be discussed aiming at a higher productivity.

Chapter 3 - The resistance acquired by pathogenic microorganisms and the consequent inefficiency of antibiotics due to prolonged use are the main problems facing medicine today. Given this, numerous researches have been designed to look for new agents with antibacterial activity, including natural products. Thus, this study aimed to evaluate the antimicrobial activity of the essential oil of *Campomanesia aurea* O. Berg (EOCA) leaves, as well as to verify its antibiotic modulating potential. The essential oil was obtained by hydrodistillation in a Clevenger type device, with 0.17% content and the identification of the chemical compounds was done in a Mass Spectrometry Coupled Gas Chromatography (GC/MS) apparatus, where the majority compounds were obtained. The chemical compounds were made in a Mass Spectrometry Coupled Gas Chromatography (GC/MS) apparatus, where the major compounds were khusimol (11.7%) and epizizizone (8.7%). Antimicrobial activity was performed by the microdilution method to determine Minimum Inhibitory Concentration (MIC), with MICs of 101.59 and 256 μg/mL for *Staphylococcus aureus* 25923 and 10 respectively. The modulating effect of antibiotics was performed by combining the EOCA with the antibiotics Ampicillin, Gentamicin and Norfloxacin against the multidrug-resistant bacteria *Escherichia coli* 06, *Staphylococcus aureus* 10 and *Pseudomonas aeruginosa* 24, where an antagonistic effect with gentamicin front *S. aureus* was observed and synergism with norfloxacin and ampicillin antibiotics for *S. aureus* 10 and *P. aeruginosa* bacteria 24. There are still few studies analyzing the modulating activity of *C. aurea*, which is the first report of this activity.

Chapter 4 - Lorem Studies with *E. intermedium*, the focus of this study, are still scarce, especially those addressing the chemical composition of its oils and extracts, or its biological, toxicological and pharmacological

activities. This study aims to investigate the chemical composition of compounds from the E. intermedium leaf essential oil, in addition to its modulatory and antibacterial action. (E)-caryophyllene, α-humulene, germacrene D, bicyclogermacrene, spathulenol, elemicin and caryophyllene oxide were the main constituents found in the EIEO, with germacrene D being the most expressive in terms of percentage (17.2%). The EIEO demonstrated significant antibacterial effect only against *S. aureus* in the MIC assays, demonstrating a modulatory effect against *S. aureus* (S.A.10) and *E. coli* (E.C.06) in a synergistic manner. However, the oil acted as antagonist for *P. aeruginosa* (P.A.24), blocking the effect of ampicillin and norfloxacin. These results contribute to the discovery of new antibiotics from natural products, especially in the treatment of multiresistant bacteria. The essential oil was obtained by hydrodistillation using a Clevenger extractor. Gas chromatography coupled to mass spectrometry (GC-MS) was used for phytochemical analysis. The antibacterial and modulatory activity assays were performed using the broth microdilution method to establish the Minimal Inhibitory Concentration (MIC) as well as the oil's potentiating effect for the chosen antibiotics against three multiresistant bacterial strains: *Staphylococcus aureus*; *Escherichia coli* and *Pseudomonas aeruginosa*.

Chapter 5 - Different cultures from around the world have been using natural products for many centuries, not only to promote human health but also prevent the development of various chronic diseases. A large body of evidence clearly demonstrates the medicinal value of a number of phytochemicals derived from these natural products. These phytochemicals have been extensively investigated for their role in disease modulation and progression. One such natural product is sandalwood oil that has been used as a traditional medicine for treating different ailments including cancer. Sandalwood oil constitutes alpha-santalol, a sesquiterpene that has been studied for its health benefits and its ability to modulate different signaling pathways involved in the development of a malignancy. For example, the antitumor and cancer preventive properties of alpha-santalol have been shown to involve cell death induction through apoptosis and cell cycle arrest in various cancer models. Alpha-santalol also decreased inflammatory markers associated with neurodegenerative and skin disorders. This chapter

summarizes the biological aspects attributed to the medicinal benefits of sandalwood oil and alpha-santalol against various ailments with relevant clinical evidence.

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Chapter 1

EDIBLE AND NON-EDIBLE OILS OBTAINED FROM PLANTS: SOURCES AND CHARACTERIZATION

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ABSTRACT

Vegetable oils are obtained from oilseeds, fruits, or nuts by different pressing methods, solvent extraction or a combination of these methods. The main components of vegetable oils are saturated and unsaturated fatty acids. The yields, different compositions, and physicochemical properties determine the oil usefulness in various applications aside edible uses, such as in foods, pharmaceuticals, cosmetics and production of energy. Economic development and populational growth have boosted the demand for vegetable oils. The chapter includes plant sources, extraction methods, physicochemical characterization and commercial application of edible and non-edible vegetable oils. Patent documents about vegetal oils recorded in Espacenet and WIPO Patentscope databases, as well as the countries, applicants and International Patent Classification (IPC) with the main records and relative percentage in WIPO Patentscope are presented. At the end of the chapter, future outlooks on the subject are provided.

Keywords: vegetable oils, exrtraction methods, characteristics

1. INTRODUCTION

Economic development and population growth have increased the worldwide demand for vegetable oils. Besides the food industry, the biofuels market deserves attention when it comes to the use of these oils. Vegetable oils are one of the main products extracted from plants, which are mainly water-insoluble (hydrophobic). They are liquid at room temperature, mainly due to the presence of double bonds between carbons in the fatty acids that compose them. They are mainly presented as triglycerides formed by the union of fatty acid molecules to the three glycerol hydroxyl groups by esters bonds (Taiz et al. 2017). Still, free fatty acids are present in smaller proportions, which normally are resulted from hydrolysis of triglycerides, glycolipids, sterols, tocopherols, phospholipids, carotenoids, and vitamins (Martins, Mello, and Suarez 2013).

The fatty acids have a numerical abbreviation, where the number before the colon is the total number of carbons and the number after the colon is the number of double bonds (Taiz et al. 2017). The main fatty acids present in vegetable lipids are lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3). The proportion of fatty acids in plant lipids depends on the species used in the extraction. For example, in peanut oil there is (mass basis) 9% of palmitic acid, 59% of oleic acid, and 21% of linoleic acid. The cottonseed oil consists of 25% palmitic acid, 15% of oleic acid, and 55% of linoleic acid (Taiz et al. 2017). A common feature among vegetable oils is the higher amount of unsaturated fatty acids.

Vegetable oils are an important form of reduced carbon storage in many seeds such as soybeans, sunflowers, canola, peanuts, and cotton. In addition to these agronomically important species, vegetable oils also occur in untamed plants and are produced by gluconeogenesis, which is the synthesis of sugars from organic acids. In most cases, they are stored in the cotyledon cell cytoplasm or endosperm, called oleosomes (Taiz et al. 2017).

The main sources of vegetable oils are seeds (oilseeds) and fruit pulp. The United States Department of Agriculture (USDA) highlighted in 2019 the production of vegetable oils from palm, soybean, canola, sunflower, peanut, cotton, coconut, and olive. However, many other species have oils in their constitution, which some of them are covered in this chapter.

The oil production varies according to the place and year of crop production because they come from different plant species and the extraction method also interferes with the worldwide production of vegetable oils. There is no single process of extracting vegetable oils because it depends on the characteristics of the oil source (Ramalho and Suarez 2013). However, it is possible to identify some basic operations involved in extracting: pressing, solvent extraction or a combination of these methods. In 2012/2013, the production and consumption were 161.72 e 159.34 million tons, respectively, while for 2018/2019 a production of 203.93 million tons and a consumption of 198.66 million tons were expected (USDA 2019).

Vegetable oils are widely used in human food, being employed as ingredients or in cooking food by frying. They are also used in the production of paints, soaps, biofuels, cosmetics, sanitizers and solvents (Martins, Mello, and Suarez 2013; Ramalho and Suarez 2013), and in herbal medicine (Cunha et al. 2012). In this sense, vegetable oils may be edible or non-edible oils.

Non-edible oils differ from edible oils in their constitution, for presenting toxic substances, like ricin in castor oil. Therefore, they should not be used for food. Examples of such oils are those extracted from jatropha and neem and are easily found in developing countries (Demirbas 2009).

Based on this context, this chapter is intended to present plant sources, extraction methods, characterization, and commercial application of edible and non-edible vegetable oils. Patent documents related to the theme available in Espacenet and WIPO Patentscope databases and the countries, as well as applicants and International Patent Classification (IPC) with the most records and relative percentage in WIPO Patentscope, are presented. Future outlooks on the subject are provided as well.

2. EDIBLE OILS

There are many vegetable sources for extracting edible oils. The USDA informs the commonly production of edible oil from palm, soybean, canola, sunflower, peanut, cotton, coconut, and olive. Expected vegetable oil production for 2018/2019 was 203.93 million tons and the consumption were 198.66 million tons (Tables 1 and 2). Vegetable oil sources are mostly seeds. However, fruit pulp is also a rich oil source. In this chapter, we chose to address the vegetable sources from coconut, cottonseed, olive, palm, peanut, rapeseed, soybean and sunflower seed due to the emphasis given by USDA, according to Tables 1 and 2.

Table 1. World vegetable oils production 2012/2013 – 2018/2019, in million metric tons*

Oil	2012/ 2013	2013/ 2014	2014/ 2015	2015/ 2016	2016/ 2017	2017/ 2018	2018/ 2019
Coconut	3.62	3.38	3.37	3.32	3.41	3.59	3.63
Cottonseed	5.21	5.16	5.12	4.29	4.42	5.16	5.20
Olive	2.51	3.20	2.40	3.12	2.48	3.26	3.10
Palm	56.43	59.36	61.87	58.90	65.27	70.46	73.49
Palm kernel	6.66	7.07	7.31	7.01	7.62	8.32	8.57
Peanut	5.40	5.72	5.43	5.44	5.77	5.95	5.57
Rapeseed	25.69	27.28	27.62	27.86	27.54	27.86	27.96
Soybean	43.33	45.24	49.32	51.60	53.72	55.17	56.97
Sunflowerseed	12.87	15.45	14.92	15.38	18.18	18.23	19.45
Total	161.72	171.86	177.36	176.92	188.41	197.97	203.93

*Source: USDA, Economic Research Service using data from USDA, Foreign Agricultural Service, Oilseeds: World Markets and Trade. Last updated: March 29, 2019.

Table 2. World vegetable oils consumption 2012/2013 – 2018/2019, in million metric tons*

Oil	2012/ 2013	2013/ 2014	2014/ 2015	2015/ 2016	2016/ 2017	2017/ 2018	2018/ 2019
Coconut	3.75	3.34	3.29	3.26	3.15	3.45	3.50
Cottonseed	5.21	5.09	5.06	4.40	4.39	5.12	5.15
Olive	2.83	2.96	2.65	2.82	2.59	2.87	3.07
Palm	55.76	57.77	58.72	59.23	61.91	66.35	71.02
Palm kernel	6.36	6.58	7.22	6.81	7.21	7.80	8.06
Peanut	5.44	5.68	5.50	5.44	5.63	5.95	5.52
Rapeseed	24.28	26.16	27.33	28.37	28.90	28.60	28.39
Soybean	42.60	45.26	47.84	52.17	53.46	54.51	56.23
Sunflowerseed	13.12	14.15	14.11	15.17	16.59	17.55	17.72
Total	159.34	166.98	171.72	177.67	183.83	192.19	198.66

*Source: USDA, Economic Research Service using data from USDA, Foreign Agricultural Service, Oilseeds: World Markets and Trade. Last updated: March 29, 2019.

2.1. Plant Sources

2.1.1. Elaeis Guineensis Jacq.

Palm oil is reddish in color, sweetish in flavor, and is extracted from the mesocarp of the palm fruit *Elaeis guineensis* Jacq., while palm kernel oil is off-white, has almost no smell and taste, and is extracted from the seed of the same species. In industrial processing, mass yield in crude palm oil is 22% and in palm kernel oil 2% (Jorge, 2009).

Palm oil is the most produced and most consumed vegetable oil in the world, with an estimated production of 73.49 and a consumption of 71.02 million tons for the 2018/2019 crop. The estimated production of palm kernel oil is 8.57 and the consumption is 8.06 million tons of palm kernel oil (USDA, 2019).

According to the USDA, palm oil and palm kernel oil production estimates for 2019 in Indonesia stands out with 43 million tons of palm oil and more than 4 million tons of palm kernel oil. Malaysia is the second largest producer of palm oil and palm kernel oil, with 21 and 2.38 million tons, respectively (Tables 3 and 4).

Table 3. Production of palm oil:
Top 10 worldwide producers*

Rank Country	Production in million metric tons
Indonesia	43
Malaysia	21
Thailand	3
Colombia	1.680
Nigeria	1.015
Guatemala	852
Ecuador	630
Papua New Guinea	630
Honduras	580
Brazil	540

*Source: USDA, 2019. Year of Estimate: 2019.

Table 4. Production of palm kernel oil: Top 10 producers*

Rank Country	Production in million metric tons
Indonesia	4.894
Malaysia	2.384
Thailand	397
Nigeria	330
Colombia	154
Guatemala	82
Papua New Guinea	75
Honduras	65
Brazil	64
Ecuador	58

*Source: USDA, 2019. Year of Estimate: 2019.

2.1.2. Glycine max (L.) Merr.

Soybean oil (*Glycine max* (L.) Merr.) has emerged as a byproduct of soybean meal processing and has become one of the world leaders in the oil market (Jorge, 2009). It is a clear and slightly yellowish oil with a characteristic mild odor and taste (FiB 2014). Soybean seeds contain approximately 20% oil (Wilson, 2004).

Table 5. Production of soybean oilseed: Top 10 producers*

Rank Country	Production in million metric tons
Brazil	123
United States	96.615
Argentina	53
China	17.100
Paraguay	10.200
India	9
Canada	6
Russian Federation	4.300
Ukraine	3.700
Bolivia	2.900

*Source: USDA, 2019. Year of Estimate: 2019.

Among the ten major countries that produce soybeans, Brazil stands out with an expected production of over 123 million tons and the United States with an expected production of over 96 million tons in 2019 (Table 5).

According to the USDA, the production in 2018/2019 was expected to reach 56.97 million tons and consumption to 56.23 million tons of the product. China is estimated to be a leader in oil production, with production expected to be over 15 million tons and the United States with more than 11 million tons of this oil (Table 6).

Table 6. Production of soybean oil: Top 10 producers*

Rank Country	Production in million metric tons
China	15.142
United States	11.077
Argentina	8.650
Brazil	8.385
Europe Union	3.021
India	1.440
Mexico	1.065
Russian Federation	896
Paraguay	739
Egypt	656

*Source: USDA, 2019. Year of Estimate: 2019.

2.1.3. Brassica napus L.

Rapessed oil (*Brassica napus* L.) is extracted from seeds that are small, round and can be yellow, brown or black, with oil content between 40 and 60% (Jorge, 2009). This oil has yellowish color with a characteristic flavor and odor (FiB 2014).

According to the USDA data, it was expected for the 2018/2019 crop production of 27.96 and consumption of 28.39 million tons. In the ranking of the ten countries with the highest estimated production of rapeseed oilseed, the leader is Canada with an expected production of over 19 million tons for 2019, followed by Europe Union with an estimated 17 million tons (Table 7). Meantime in the ranking of the ten largest producers of rapeseed oil, Europe Union leads with an expectation of more than 9 million tons of

the product and China was expected to produce more than 6 million tons of this oil (Table 8).

Table 7. Production of rapeseed oilseed: Top 10 producers*

Rank Country	Production in million metric tons
Canada	19.500
Europe Union	17
China	13.100
India	7.700
Ukraine	3.300
Russian Federation	2.100
Australia	2.100
United States	1.693
Belarus	490
Kazakhstan	320

*Source: USDA, 2019. Year of Estimate: 2019.

Table 8. Production of rapeseed oil: Top 10 producers*

Rank Country	Production in million metric tons
Europe Union	9.154
China	6.240
Canada	4.225
India	2.584
Japan	1.080
Unites States	765
Mexico	580
Russian Federation	558
Pakistan	395
Australia	310

*Source: USDA, 2019. Year of Estimate: 2019.

2.1.4. Helianthus annuus L.

The oil extracted from sunflower seeds (*Helianthus annuus* L.) is clear and light golden yellow, with a characteristic mild odor and taste (FiB 2014). Sunflower seed is composed of approximately 47.3% of grease in its composition (Jorge, 2009).

According to the USDA, it was expected to produce 19.45 million tons and consume 17.72 million tons for the 2018/2019 crop. Ukraine is the worldwide leader in sunflower seed oilseed and sunflower seed oil production, with an estimated production of over 14 million tons and more than 6 million tons respectively, followed by the Russian Federation, which the production is approximately 13 million tons of sunflower seed oilseed and more than 5 million tons of sunflower seed oil (Table 9 and Table 10).

Table 9. Production of sunflower seed oilseed: Top 10 producers*

Rank Country	Production in million metric tons
Ukraine	14.500
Russian Federation	13
Europe Union	9.800
Argentina	3.500
China	3.250
Turkey	1.750
United States	1.022
Republica of Maldova	900
Kasakhstan	800
South Africa	750

*Source: USDA, 2019. Year of Estimate: 2019.

Table 10. Production of sunflower seed oil: Top 10 producers*

Rank Country	Production in million metric tons
Ukraine	6.149
Russian Federation	5.162
Europe Union	3.739
Argentina	1.365
Turkey	935
China	735
South Africa	335
Serbia	234
Kasakhstan	209
United States	200

*Source: USDA, 2019. Year of Estimate: 2019.

2.1.5. Arachis hypogaea L.

Table 11. Production of peanut oilseed: Top 10 producers*

Rank Country	Production in million metric tons
China	17.500
India	5.200
Nigeria	3.500
United States	2.560
Sudan	1.800
Myanmar	1.375
Argentina	1.255
Senegal	1.100
United Republic of Tanzania	1.100
Indonesia	990

*Source: USDA, 2019. Year of Estimate: 2019.

Table 12. Production of peanut oil: Top 10 producers*

Rank Country	Production in million metric tons
China	2.944
India	1.125
Myanmar	270
Nigeria	265
Sudan	230
United Republic of Tanzania	138
United States	106
Argentina	106
Burkina Faso	80
Senegal	72

*Source: USDA, 2019. Year of Estimate: 2019.

Peanut *(Arachis hypogaea L.)* seeds contain approximately 44 to 56% oil (Gulluoglu et al. 2016). The oil has a pale yellow color, characteristic odor, and mild taste (FiB 2014). According to the USDA data, the estimated peanut oil production for 2018/2019 was5.57 and the consumption was 5.52 million tons (USDA, 2019). In the ranking of peanut oilseed and peanut oil production estimates for 2019, China is the worldwide leader in production, with an estimated production of over 17 million tons of peanut oilseed and nearly 3 million tons of peanut oil. India is the second largest producer, with

an estimated 2019 production of 5.2 and 1.125 million tons of peanut oilseed and peanut oil, respectively (Tables 11 and 12).

2.1.6. Gossypium hirsutum L.

Cotton seeds (*Gossypium hirsutum* L.) contain between 15 and 25% of edible oil (Bhattacharjee, Singhal, and Tiwari 2007). Cotton oil has a slight chestnut flavor and light golden to reddish-yellow coloring, which varies with the degree of refinement (FiB 2014).

Table 13. Production of cottonseed oilseed: Top 10 producers*

Rank Country	Production in million metric tons
India	12.737
China	10.679
United States	5.854
Brazil	3.798
Pakistan	3.032
Turkey	1.241
Uzbekistan	1.175
Europe Union	594
Mexico	537
Benin	479

*Source: USDA, 2019. Year of Estimate: 2019

Table 14. Production of cottonseed oil: Top 10 producers*

Rank Country	Production in million metric tons
India	1.400
China	1.356
Brazil	592
Pakistan	430
United States	243
Turkey	221
Uzbekistan	185
Mexico	96
Australia	75
Mali	59

*Source: USDA, 2019. Year of Estimate: 2019.

The USDA reports that the estimated production of cottonseed oil for 2018/2019 was 5.20 and the consumption was 5.15 million tons. India is the worldwide largest producer of cottonseed oilseed and cottonseed oil, with production expected to be over one million tons by 2019. China is expected to produce another 10 million tons of cottonseed oilseed and more than 1 million tons of cottonseed oil, ranking second in the 2019 production estimate (Table 13 and Table 14).

2.1.7. Cocos nucifera L.

Coconut oil is extracted from fresh coconut pulp *(Cocos nucifera L.)* and its yield is approximately 62.4%. This oil solidifies below 25°C and it is considered an extra virgin oil because it has an acidity index of less than 0.5% (FiB 2014).

Table 15. Production of coconut oil: Top 10 producers*

Rank Country	Production in million metric tons
Philippines	1.638
Indonesia	980
India	475
Viet Nam	184
Mexico	139
Sri Lanka	44
Thailand	31
Malaysia	21
Côte D'ivoire	20
Papua New Guinea	14

*Source: USDA, 2019. Year of Estimate: 2019.

According to the USDA, the estimated production of coconut oil for the 2018/2019 crop was 3.63 million tons, while the consumption was approximately 3.50 million tons. Among the producing countries, the Philippines stands out with an estimated production of 1.698 million tons of coconut oil, followed by Indonesia with a production of almost 1 million tons for 2019 (Table 15).

2.1.8. Olea europaea L.

Pressing the pulp of Olea europea L. (olive) provides about 25 to 30% of olive oil. This oil is called olive oil because it is extracted from the endocarp (FiB 2014). The USDA estimated that in the 2018/2019 the harvest olive oil production was 3.10 and consumption was 3.07 million tons. Noteworthy, in Europe Union, the production is about 2.310 million tons of this oil (Table 16).

Table 16. Production of olive oil: Top 10 producers*

Rank Country	Production in million metric tons
Europe Union	2.310
Tunisia	290
Turkey	250
Morocco	140
Syrian Arab Republic	105
Algeria	80
Argentina	38
Lebanon	25
Jordan	24
Australia	22

*Source: USDA, 2019. Year of Estimate: 2019.

2.2. Extraction Methods

Edible vegetable oils are basically extracted by pressing or using a solvent. Generally, oilseeds and other fatty materials with oil contents below 25% are subjected to solvent extraction of the oil. Materials with oil content higher than 25% are previously pressed to obtain a castor cake with 10 to 15% oil content, which is then extracted using a solvent. Among the solvents used, hexane is the most common in the process of extracting oil from plants (Jorge 2009).

However, studies are conducted to improve the extraction of vegetable oils, such as: using supercritical carbon dioxide (Bhattacharjee, Singhal, and Tiwari 2007), using propane, ethanol and its mixtures as compressed solvent (Jesus et al. 2013), using surfactant microemulsion-based oil seed extraction

(Naksuk, Sabatini, and Tongcumpou 2009), using enzyme-assisted aqueous extraction processing (Wu, Johnson, and Jung 2009), and using supercritical fluid extraction (Jokić et al. 2010; Sovilj 2010; Prado et al. 2012; Martínez and Aguiar 2013; Balvardi et al. 2015; Ben Rahal et al. 2015).

2.3. Oil Composition

2.3.1. Palm Oil and Palm Kernel Oil

Palm oil is one of the richest natural sources of carotenoids, with concentrations ranging from 700 to 1000 ppm, mainly formed by beta carotenes and alpha carotenes, besides being rich in vitamin E (tocopherols and tocotrienols) (FiB 2014). Palm oil consists of about 50% saturated fatty acids, 40% monounsaturated fatty acids and 10% polyunsaturated fatty acids. The main fatty acids found in palm oil are: palmitic acid (41.8–46.8%), oleic acid (37.3–40.8%), linoleic acid (9.1–11%), stearic acid (4.2 - 5.1%), myristic acid (0.9 - 1.5%) and linolenic acid (0.4%) (Jorge 2009).

Palm kernel oil has in its constitution lauric acid (48%), myristic acid (16%) and palmitic acid (8%) totaling 82% of saturated fatty acids. Oleic (15%) and linoleic (3%) acids also occur, which are unsaturated fatty acids and comprise 18% of the constitution of this oil (Jorge 2009).

2.3.2. Soybean Oil

Soybean oil is high in linoleic acid (omega 6), in addition to oleic acid (omega 9) and linolenic acid (omega 3) (FiB 2014). It consists of essential fatty acids such as linoleic (35 - 60%) and linolenic (2.0 - 13.0%), which are unsaturated fatty acids. Also present in soybean oil are the tocopherols, with a vitamin character and antioxidant activity (Jorge 2009).

2.3.3. Rapeseed Oil

Rapeseed oil has 2.5 to 6.5% palmitic acid, 0.8 to 3.0% stearic acid, 53.0 to 70% oleic acid, 15 to 30% linoleic acid and 5 to 13% of linolenic acid (Jorge 2009). It is one of the healthiest due to the high amount of Omega-3, vitamin E, monounsaturated fats and the lowest fat content of all vegetable

oils (Cardoso et al. 2010). Among the edible oils, this has the lowest saturated fatty acid content (7%), high monounsaturated content (61%), and 32% of polyunsaturated content, with 11% of alpha-linoleic acid (Omega-3) (FiB 2014).

2.3.4. Sunflower Seed Oil

Sunflower seed is rich in oleic acid (49.02%), linoleic acid (45.35%), palmitic acid (4%), and stearic acid (1.47%) (Correia et al. 2014). It is an oil-rich in vitamin E and has approximately 10% of saturated fatty acids (Jorge 2009; FiB 2014).

2.3.5. Peanut Oil

Peanut oil has 50 to 60% of oleic acid, 18 to 30% of linoleic acid, 6 to 12% of palmitic acid, and practically no linolenic acid. The presence of arachidic acid is characteristic of peanut oil and it is used to identify the presence of peanut oil in mixtures with other oils such as olive oil (Jorge 2009). In addition, it contains high vitamin E content (FiB 2014).

2.3.6. Cottonseed Oil

Cottonseed oil contains a mixture of saturated and unsaturated fatty acids, the main component being linoleic acid (Omega-6) and oleic acid (Omega-9), is rich in palmitic acid, between 22 and 26%, oleic acid, between 15 and 20%, and linoleic acid, between 49 and 58% (Jorge 2009; FiB 2014).

2.3.7. Coconut Oil

Coconut oil has a maximum acidity index of 0.5% and it is characterized as an extra virgin oil (FiB 2014). It consists of more than 82.2% of saturated fatty acids, in particular lauric acid (41%) and myristic acid (20.3%). In a smaller proportion, there are caprylic (4.7%), capric (4.1%), palmitic (12.3%), oleic (9.9%) and linoleic (3.6%) acids. These values may change according to the genetic material used, production system adopted, age of the fruits harvested, soil conditions and climate at the planting site (Correia et al. 2014).

2.3.8. Olive Oil

Olive oil contains about 90% of unsaturated fatty acids, the main component being oleic acid. It has a low level of polyunsaturated fatty acids, approximately 10%. It is a source of vitamin E and polyphenols, containing about 100 mg/kg of vitamin E and 300 mg/kg of polyphenols (Jorge 2009). It has 8% of linoleic acid and <1% of linolenic acid (FiB 2014).

2.4. Commercial Application

Vegetable oils obtained from different vegetable sources have several applications in the industry, including the food, cosmetic and biofuel production areas. Some applications are summarized in Table 17.

Table 17. Vegetable species and oil applications

Vegetable species	Applications
Palm	Biodiesel[1, 8], metal cutting fluid[2], culinary[12]
Soybean	Biodiesel[3, 6, 7], culinary[12], cosmetics and skin care[13]
Rapessed	Biodiesel[4, 7], metal cutting fluid[2], culinary[12], cosmetics and skin care [13]
Sunflower seed	Biodiesel[5,6,7], culinary [12], cosmetics and skin care [13]
Peanut	Biodiesel[6], metal cutting fluid[2], culinary[12], cosmetics and skin care[13]
Cottonseed	Biodiesel[8, 9], cosmetics and skin care[13]
Coconut	Biodiesel[10], metal cutting fluid[2], culinary[12], cosmetics and skin care[13]
Olive	Biodiesel[11], culinary[12], cosmetics and skin care[13]

[1] (Salamanca et al. 2012); [2] (Naik et al. 2010); [3] (Allami et al. 2019); [4] (Silva et al. 2017); [5] (ANTOLÍN et al. 2002); [6] MAPA 2006; [7] MAPA 2015; [8] (Rashid, Anwar, and Knothe 2009); [9] (Nabi, Rahman, and Akhter 2009); [10] (Yuliansyah, Triwidagdo, and Abuhasan 2019); [11] (Uyumaz, BOZ, and BAYDIR 2017); [12] (Foster, Williamson, and Lunn 2009); [13] (Athar and Nasir 2005).

3. NON-EDIBLE OILS

As commented earlier, vegetable oils are mainly extracted from seeds and are characterized by a complex mixture of chemical compounds, with fatty acids predominating. There is variation in the composition of vegetable oils, even within the same species, mainly by different cultivation

conditions, edaphoclimatic conditions, and genetic variability. Unlike edible oils, non-edible oils are classified as having toxic and/or allergenic substances in their constitution, so they should not be used for human consumption (Demirbas 2009). Ricin, present in castor oil (*Ricinus communis* L.), is an example of a toxic compound present in vegetable oil.

Non-edible oils are an important source of income and drive the economy of emerging countries compared to the production of edible oils (Demirbas 2009). Typically, non-edible oil producing plants are adapted to arid and semi-arid conditions and require low soil fertility. In addition, these plants do not compete with food producers, so seed residues after oil extraction, called castor cake, can be used as fertilizer for soil enrichment (Azam, Waris, and Nahar 2005).

As they cannot be used as food, as its name says non-edible vegetable oils, they have other uses, depending on the species that is extracted and the extraction process. They are used as biocides and are an important alternative source for the production of renewable fuels as an alternative to petroleum. Numerous studies with different oilseed species are being conducted in search of a non-edible source for the production of good quality biodiesel.

3.1. Plant Sources

There are many vegetable sources for extracting non-edible oils. Non-edible oil is produced from a number of plant species, including castor oil (*Ricinus communis* L.), Jatropha (*Jatropha curcas* L.), neem (*Azadirachta indica* A. Juss.), rubber tree (*Hevea brasiliensis* L.), karanja (*Pongamia pinnata* (L.) Pierre) and Alexandria Bay (*Calophyllum inophyllum* L.), tobacco (*Nicotiana tabacum* L.), pequi (*Caryocar brasiliense* Camb.), moringa (*Moringa oleifera* L.), crambe (*Crambe abyssinica* Hochst.), *Cerbera odollam* Gaertn., *Balanites aegyptiaca* Del., jojoba (*Simmondsia chinensis* (Link) C.K. Schneider), among others. The oil extracted from these plants is the primary product, and the extraction residue, commonly

called cake, is a byproduct that can be used as organic fertilizer in soil enrichment.

Although there are many promising species for the production of non-edible oils, there is virtually no official data on the production of these oilseed species. In the following, we will discuss in more detail some common non-edible oil-producing species.

3.1.1. Ricinus communis L.

The species *Ricinus communis*, popularly known as castor oil, belonging to the family Euphorbiaceae, is originally from Africa (Ventura 1990). It is a shrub plant with high oil production capacity, being undemanding about the soil and quite adaptable, and has become an alternative to meeting biofuel production programs, mainly in places like the semiarid in Brazil (Machado et al. 1998) (Ogunniyi 2006; Mubofu 2016).

India is the main producer and accounts for over 75% of total production followed by China and Brazil, accounting for 12.5 and 5.5% respectively (Severino et al. 2012). Data from Miragaya (2005), showed that castor bean yielded an average yield of 1.5 tons of seed per hectare, with an average oil content of 48%, resulting in oil production of approximately 720 kg/ha.

3.1.2. Jatropha curcas L.

The species *Jatropha curcas*, popularly known as Jatropha, is a shrubby plant belonging to the family Euphorbiaceae. Its origin is controversial, but several authors cite Central America as likely center of origin. Currently, it is naturally found in the tropical regions of the world, including in Asia (Giibitz, Mittelbach, and Trabi 1999; Arruda et al. 2004). It is a very rustic plant, undemanding about soil nutrients, tolerant to water deficit and able to be used in the recovery of degraded areas due to its deep roots (Teixeira, 2005; Kobayasti et al. 2011). On the other hand, these same characteristics that contribute to a good adaptation of *Jatropha curcas* also limit its cultivation. For example, continuous flowering and heterogeneous fruit ripening (Rocha et al. 2019).

Despite its great potential for production, its oil is non-edible because it has toxic compounds, like curcin and phorbol esters (Makkar et al. 1997;

Martínez-Herrera et al. 2006). It is also present in the castor cake that is usually generated as oil extraction residue (Virgens et al. 2017). This characterizes a disadvantage of this species compared to other oil-producing species. Recently, another compound with allergenic potential has been identified, a protein similar to castor 2S albumin (Martı´nez-Herrera et al. 2006). The species *Jatropha curcas* ends up being mainly cultivated for the production of fuel substitute oil (Giibitz, Mittelbach, and Trabi 1999).

Jatropha oil is extracted from seeds by pressing followed by solvent extraction or directly by solvent extraction using Soxhlet. Reports show that the use of yellowish-brown fruits, in addition to providing a maximum oil extraction yield, also allows obtaining a lower acidity oil and a better quality for industrial purposes (Santos 2011).

According to information from Tominaga et al. (2007), the plant produces on average 100, 500, 2,000 and 4,000 grams of seed per plant in the first, second, third and fourth years of cultivation, respectively. Productivity can still vary with spacing, up to 6,000 kg/ha of seeds. Thus, with this productivity, it would be possible to produce around 2,000 kg/ha of oil, a value higher than the production potential soybean oil, which is of 500 kg oil/ha (Gonçalves, Mendonça and Laviola 2009). However, it is believed that with further studies on breeding systems improvement and genetic improvement, the jatropha can produce above 4,000 kg/ha of oil (Laviola and Dias 2008). Reports show that the use of yellow-brown fruits, in addition to providing maximum oil extraction yield, also allows for a lower oil quality and better quality for industrial purposes (Santos 2011).

3.1.3. Azadirachta indica A. Juss.

Neem is a leafy tree belonging to the Meliaceae family, originating in India and Myanmar, growing in tropical and semitropical regions (Demirbas 2009). Neem crops around the world, mainly in South Asia and sub-Saharan Africa, are estimated to produce between 0.7 to 3.2 and 1 to 4.6 million tonnes of fruit respectively per year (Nde, Boldor, and Astete 2015).

Neem vegetable oil is obtained by pressing the fruits and seeds. Neem is very sensitive to cold but withstands well droughts and high temperatures. Thus, in warmer and drier places, the species presents good fruit/seed

production for oil extraction. The fruit is a 1.5 to 2 cm long oval berry with white skin and yellowish flesh. The neem tree is reported to produce about 40 to 50 kg of fruit per plant per year, equivalent to 25 to 30 kg of seeds (Djibril et al. 2015).

3.2. Extraction Methods

For the extraction of vegetable oils, there is no single process as it will depend on the characteristics of the raw material from which the oil is to be extracted. As with edible vegetable oils, non-edible oils are extracted primarily by pressing, pressing followed by solvent extraction or directly by solvent extraction using Soxhlet. The latter method consists of leaching the oil contained in the test material through contact cycles with a particular solvent, usually n-hexane, ethanol or methanol. There is also oil extraction by supercritical fluid (Reverchon and Marrone 2001).

3.3. Oil Composition

3.3.1. Castor Oil

Castor oil is extracted by one or a combination of mechanical pressing and solvent extraction. The extraction of oil is initially performed by mechanical pressing of crushed seeds and is completed in Soxhlet extractor using solvents, being heptane, hexane and petroleum esters the most used (Ogunniyi 2006). In general, the oil obtained from castor seed is a thick, very viscous liquid, the color of which varies from colorless to dark yellow, with varying smell and taste, sometimes very unpleasant and nauseating.

A castor oil yield of around 45 to 50% is reported (Koh, Idaty, and Ghazi 2011). However, a large number of castor varieties in existence make the oil contents vary from 44 to 55% of the dry mass of the seeds (Paes, Souza, and Lima 2015). The resulting castor oil is viscous, light odor, pale yellow and non-volatile (Ogunniyi 2006). A specific feature of castor oil is the presence of ricin oleic acid, also called ricin. Table 18 shows some of these variations

in the percentage of major fatty acids present in castor oil, according to literature (Pinto et al. 2005; Mubofu 2016).

Table 18. Approximate composition of the major fatty acids present in the vegetable oil of *Ricinus communis* L. (castor bean)

Fatty acid	Percentage (%)
Ricin oleic (C18:1)	74.1 – 89.5
Linoleic (C18:2)	1.2 – 10.32
Oleic (C18:1)	3.0 – 7.55
Palmitic (C16:0)	1.0 – 3.0
Stearic (C18:0)	1.0 – 3.0

3.3.2. Jatropha Oil

The yield of oil extracted from *Jatropha curcas* seeds can vary from 50% to 60%, higher compared to castor oil (Koh, Idaty, and Ghazi 2011). The fatty acids in jatropha oil are mostly unsaturated (70.5%). Other authors report different yields, which may occur due to the cultivars used, the seed maturity stage and the extraction process used (Virgens et al. 2017). The composition of the major fatty acids present in the oil may also vary, and Table 19 shows some of these variations, according to literature (Akbar et al. 2009).

Table 19. Approximate composition of fatty acids present in *Jatropha curcas* L. vegetable oil

Fatty acid	Percentage (%)
Linoleic (C18:2)	32.8
Oleic (C18:1)	25.7
Palmitic (C16:0)	14.2
Stearic (C18:0)	7.0

3.3.3. Neem Oil

From the neem oil three active ingredients have been isolated: Nimbim (0.1%), Nimbinim (0.01%) and Nimbidim (0.1%). Also, other different

substances neemola, margosin, totradecoic acid and an acid called D are found (Neves et al. 2003).

The oil has a high azadiractin content, which is used as a raw material in the manufacture of fungicide and insecticide products, as a natural repellent for use in veterinary medicine (Martinez 2002), as well as the production of toothpaste, soaps, among others (Neves and Carpanezzi 2009). Table 20 shows some of these variations in the percentage of fatty acids in neem oil, according to literature (Azam, Waris, and Nahar 2005).

Table 20. Approximate composition of the fatty acids present in the vegetable oil of *Azadirachta indica* A. Jass. (neem)

Fatty acid	Percentage (%)
Linoleic (C18:2)	7.5
Oleic (C18:1)	61.9
Palmitic (C16:0)	14.9
Stearic (C18:0)	14.4

3.4. Commercial Application

Non-edible vegetable oils, as they cannot participate in human food, are used for other purposes.

3.4.1. Castor Oil

Castor oil, due to its high stability and viscosity, can be used in several industrial sectors, such as manufacturing of antifreeze products used in aircraft fuels (Carvalho 1991), nylon and plastic material manufacturing (Freire 2001), and sustainable production of biodiesel (Freitas and Fredo 2005; Ijaz et al. 2016). Castor oil is rich raw material for sustainable biodiesel production (Ijaz et al. 2016). Ricin in the oil, although highly toxic, has been tested for therapeutic use in chemotherapy as well as in stem cell research (Bies, Lehr, and Woodley 2004).

3.4.2. Jatropha Oil

Potential products in the *Jatropha curcas* production chain are biodiesel, aviation biokerosene, and glycerin, which can be marketed crude or purified (Richetti and Santos 2019). In addition, there is a wide use in the production of biocides in general (Shanker and Dhyani 2006).

3.4.3. Neem Oil

Neem oil is a very eclectic product, having numerous uses. It is widely used in toiletries such as shampoos and conditioners, as well as a hair tonic, moisturizing lotions and hair and nail oil (Soares et al. 2006). In addition, neem oil is an important raw material in the manufacture of pest control products such as insects and fungi (Neves et al. 2003; Tofel et al. 2017), and also has anticarcinogenic and contraceptive effects (Girish and Shankara 2008; Ortega and Campos 2019). In addition, neem oil can also be used in the manufacture of biodiesel.

4. PATENTS SURVEY

Patent data for vegetable oil are extensive and comprehensive, addressing its different uses, species, production and associated technologies. At the same time, a patent search may provide relevant information not available in other search alternatives. Although the number of patents by itself does not provide an indication of their relative importance and impact (Haščič and Migotto 2015), data analysis and patent search can give information on trends and innovation in a specific field.

Keyword search has been carried out using the Espacenet and WIPO Patentscope. European Patent Office's Espacenet offers free access to over 117 million patent documents from over 90 countries (EPO 2019). WIPO Patentscope database provides access to 78 million patent documents including 3.7 million published international patent applications (PCT) (WIPO 2019). Only vegetable oils covered in this chapter were researched. Vegetal common name and the term 'oil' using boolean operator "AND" were applied in the search on titles, abstract or claims. The search was

conducted from December 13 to 18, 2019. Total results in both databases and available analysis in WIPO Patentscope are presented in Table 21.

Table 21. Vegetable oil total occurrences of patent documents in Espacenet and WIPO Patentscope databases and the countries, applicants and International Patent Classification (IPC) with the most records and relative percentage in WIPO Patentscope

Plant common name	Espacenet	WIPO Patentscope			
	Occurrences	Occurrences	Country	Applicant	IPC
Coconut	28,834	224,774	United States of America (37.87%)	The Procter & Gamble Company (2.39%)	A61K - (44.69%) C07D - (19.83%)
Palm	26,709	145,529	United States of America (38.07%)	The Procter & Gamble Company (2.56%)	A61K - (33,44%) A61Q - (12,88%)
Soy	27,747	139,554	United States of America (39.87%)	The Procter & Gamble Company (1.22%)	A61K -(43.17%) A23L - (18.44%)
Canola	6,758	68,983	United States of America (39.49%)	BASF SE (2.48%)	A61K - (25.37%) C12N -(21.74%)
Sunflower	23,306	145,497	United States of America (38.45%)	Unilever PLC (2.31%)	A61K - (25.37%) C12N - (15.65%)
Peanut	27,203	240,853	United States of America (38.75%)	The Procter & Gamble Company (0.91%)	A61K - (60.19%) C07D - (28.11%)
Cottonseed	13,564	145,030	United States of America (39.23%)	The Procter & Gamble Company (1.24%)	A61K - (60.78%) C07D - (28.01%)
Olive	29,693	272,502	United States of America (38.29%)	UNILEVER PLC (0.80%)	A61K - (64.51%) C07D - (26.09%)
Jatropha	1,098	6,853	United States of America (31.80%)	Exxonmobil Research and Engineering Company (2.50%)	C12N - (24.85%) C10L - (18,78%)
Castor	47,603	273,118	United States of America (39.00%)	BASF SE (1.67%)	A61K - (51.41%) C07D - (17.31%)

Table 21. (Continued)

Plant common name	Espacenet	WIPO Patentscope			
	Occurrences	Occurrences	Country	Applicant	IPC
Neem	1,314	6,734	United States of America (37.02%)	Syngenta Participations AG (5.11%)	A01N - (41.88%) A61K - (40.09%)

IPC- International Patent Classification: **A61K** - preparations for medical, dental, or toilet purposes; **C07D** - heterocyclic compounds; **A61Q** - specific use of cosmetics or similar toilet preparations; **A23L** -foods, foodstuffs, or non-alcoholic beverages, not covered by subclasses A21D or A23B-A23J; their preparation or treatment, e.g., cooking, modification of nutritive qualities, physical treatment; preservation of foods or foodstuffs, in general; **C12N** - microorganisms or enzymes; compositions thereof; propagating, preserving, or maintaining microorganisms; mutation or genetic engineering; culture media; **C10L** - fuels not otherwise provided for; natural gas; synthetic natural gas obtained by processes not covered by subclasses C10G OR C10K; liquefied petroleum gas; use of additives to fuels or fires; fire-lighters; **A01N** – preservation of bodies of humans or animals or plants or parts thereof; biocides, e.g., as disinfectants, as pesticides or as herbicides; pest repellants or attractants; plant growth regulators.

Most patents are filed in the United States of America. Companies operating in the area of personal health, personal care and hygiene, food and beverage and chemical industries in the areas of production of agrochemicals and medicines, as well as companies in the oil and gas area, hold a large number of patents of these vegetable oils. Most patents are related to preparations for medical, dental, or toilet purposes, followed by heterocyclic compounds by International Patent Classification (IPC). Patens documents address the process for recovering, devices and processes for extraction, products of different uses such as medicines, fuel, food and various other.

Patent research and its analysis have limitations as mentioned earlier. For example, the same patent is filed at different locations, thus resulting in multiple registrations. Also, the number of documents for a patent applicant may be higher because the same company comes up with different spellings and the extent to which they are duplicates or different documents is difficult to assess. In the present analysis, for example, the distinct occurrences of The Procter & Gamble Company and Procter & Gamble have not been added. Thus, the dominance of some applicants may be even higher.

5. FUTURE OUTLOOKS

The worldwide consumption of animal oils and fats is decreasing in favor of the consumption of vegetable oils, mainly due to factors such as raw material diversity, cost of production, oil extraction technologies and factors related to healthy living and eco-friendly products. However, currently, the production of plants that produce non-edible oils is still low and the use of their oils is still incipient.

The wide range of chemical compounds present in vegetable oils and the multitude of products that can be made from vegetable oils demonstrates the importance of cultivating these oilseed species, both those considered edible as well as non-edible. Several sectors (agricultural, industrial, pharmaceutical and cosmetic) can use vegetable oils as raw material, giving them a great utility value.

From the pharmaceutical and cosmetic point of view, there are wide possibilities of using non-edible vegetable oils, mainly due to characteristics such as bactericide, fungicide, insecticide, antioxidant and anticancer. From an energy point of view, since the Industrial Revolution, energy use has been continuously increasing, being mainly composed of non-renewable sources: oil, coal and natural gas. Thus, there is a growing demand for alternative and sustainable sources of energy from petroleum fuel, and vegetable oils are a great biofuel feedstock, especially for emerging countries seeking to strengthen their energy mix.

There are a variety of plant species, and every day new plant options emerge that have the potential to be used as raw materials in biodiesel manufacturing, which have characteristics such as non-toxic, biodegradability and non-emission of greenhouse gases into the atmosphere and diesel-like properties, allowing for blending (Takase et al. 2015). However, it should be considered that within the oleaginous plant species, there are those that can be consumed as food and others not, for presenting toxic and/or allergenic substances.

In Brazil, biodiesel is produced primarily from soybean oil (71.6%), the second source of raw material is animal fat (16.8%), followed by other fatty materials - including palm oil, peanut oil, turnip oil, sunflower oil, castor oil,

sesame oil and used frying oil - (11.3%), and cotton oil (0.3%) (National Agency of Petroleum, Natural Gas and Biofuels, 2018). However, there is a current that tries to set aside edible oil-producing plant species in this biodiesel production process and to use non-edible oils, since plants that produce non-edible oils do not compete with edible oil-producing plants. They are hardy and undemanding plants such as soil and climate, grow in regions that are often lacking in other crops and thus can improve the local economy and generate jobs.

Technical information on crops, especially non-edible oilseeds, is often scarce and often conflicting. In this sense, more research is required on agronomic issues, such as regions with ideal edaphoclimatic conditions, planting systems, cultural treatments, also on genetic variability and breeding, multiplication, seed productivity, and more efficient extraction methods.

In addition, better use can be made of the by-product coming from the press crushing of the beans to remove oil, called pie. In the case of cake from edible oilseeds, there is no impediment and it can be used mainly as organic fertilizer, as they are rich in potassium, nitrogen, and phosphorus, but also as animal feed, for its high protein value. On the other hand, in non-edible oilseed plants, the toxic compounds persist in the cake. In this sense, research is being carried out to detoxify the pie so that this by-product can also be used for other purposes. In the future, it is expected that edible oils will be used especially for food purposes so that this need will be met and only non-edible oils will be used by the pharmaceutical, cosmetic and biodiesel industry.

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Chapter 2

AN INSIGHT INTO EXTRACTION OF ESSENTIAL OIL USING SONIC WAVES

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ABSTRACT

Minimal or negligible side effects of secondary metabolites obtained from plant material explain the growth of the essential oil (EO) industry. Essential oils are the naturally occurring metabolites known as valuable chemicals that possess several beneficial characteristics and can replace the chief synthetic supplements. EOs are widely recognized for their aromatic, medicinal, acaricidal and antibacterial properties. These characteristic

properties have taken over a variety of synthetic medicines as natural products defined as 'generally recognized as safe products'. A variety of EOs as java citronella oil, mint oil, patchouli oil, etc. with varying composition of valuable chemicals has found its place in the industrial market of flavor, fragrance, cosmetic and medical field. This drive has increased the demand for EO sequentially in the market. The study has proven the upsurge of the natural product based cosmetic market in the United States by 9% and the United Kingdom by 8%. The commodity export from India was increased by 37%. The hydrophobic valuable compounds obtained from the plant material are volatile and can be easily extracted from various parts of plant material. However, because of their lower availability in plant material, it is important to extract them effectively by developing an enhanced technique. To avail their magnificent characteristics ultrasound-assisted extraction could be a promising option. Many researchers have extracted the essential oil using ultrasound wherein physical damage caused by cavitation bubbles leads to an increase in the productivity of essential oil. This chapter would describe the extraction of essential oil through ultrasound to increase the productivity of favorable essential oils. Later, the operation of ultrasound-assisted extraction along with mechanism will be described. A variation in sonication parameters may affect the extraction efficiency which is discussed in detail in this chapter. Also, the comparison of various techniques is made to show its environmental legibility, improved extraction in terms of extraction time, quantity and quality of natural product. Later, various optimization techniques utilized for extraction will be discussed. Also, the upscaling of the method will be discussed aiming at a higher productivity.

INTRODUCTION

The harmful impacts of drugs and synthetic materials have diverted the new era community towards natural products. The economic study has shown a rise in the market of natural products every year which evidences the awareness regarding the usage of less harmful products [1]. A minimal or negligible side effect of the metabolites obtained from plant material explains the growth of its industries enfolding essential oils (EOs) as well. The EOs are the valuable metabolites [2] which possess several beneficial characteristics and can replace the chief synthetic supplements. EO was more appreciated when scientists found its antiseptic properties with aroma

and dermal permeability. EO was revered more when many diseases were found to be cured by aromatherapy. For the past 6000 years, China, India and Egypt have skillfully used this therapy [3]. EOs are recognized for their aromatic, medicinal [4–7], acaricidal [8–10] and antibacterial [11] properties. These characteristic properties have taken over a variety of synthetic medicines [12] as natural products have been defined as 'generally recognized as safe' products. A variety of EOs as java citronella oil, mint oil, patchouli oil, etc. with varying composition of valuable chemicals has found its place in the industrial market of flavor, fragrance, cosmetic and medical field. Essential oil is a volatile complex mixture to be extracted from the various parts of a plant material.

Due to the rapid rise in interest of natural products, 300 EOs are of commercial interest out of 3000 listed EOs [13]. The global market of essential oil was US$ 7.03 Bn in 2018 and anticipated to rise to US$ 14.60 Bn by 2026. EO market is especially rising in the food and beverage sector [14]. Insecticidal, anti-parasitic and anti-toxigenic properties of EOs are some of the primary factors for their preferences in the food and beverage sector [15]. As the resultant of these properties, the EOs can increase their shelf-life and storage capability. Increasing demands of customers towards a greener and sustainable product has grown the research activities towards the extraction of essential oil to amplify the market demands. The extraction of these aromatic compounds can be done by various techniques when they are being naturally synthesized in cellular structures.

Conventionally, the EOs have been extracted by water-based classical methods. The fundamental mechanism of isolation requires either permeation of oil through the cell wall or the rupture of EO cells, residing beneath the epidermis layer. Histochemical analysis has provided detailed information regarding the localization of EO glands. Based on the characteristic of EO and plant material, various extraction techniques can be employed. For extraction, among a range of techniques, the distillation is the simplest method that is applicable for the isolation of a majority of the compounds. Major difference between the various distillation processes would be the presence of varied solvent, phases of solvent and operating conditions. The conventional extraction processes include hydrodistillation,

steam distillation and solvent extraction wherein plant material with an appropriate type and quantity of solvent is subjected to extraction for a required time period. These techniques have gained wide attention during the rising era of natural products. These techniques can extract the EO with a reasonable quantity; however the quality and time of extraction would get compromised. A longer time period for extraction can reduce the quality of EO due to the hydrolysis of water sensible compounds [16]. Also, the consumption of energy increases leading to higher emission of CO_2 because of the consumption of fuel to energy. In the era of green development, the energy consumption in terms of either electricity or coal should be reduced to lay off lesser carbon footprints. Many novel techniques were designed and developed to ensure the better extraction efficiency without compromising the quality. Ultrasound-assisted extraction (UAE) is proven to be one of the techniques for enhanced extraction of EO.

ULTRASOUND ASSISTED EXTRACTION

The UAE was developed with the purpose of following stringent government norms regarding the environmental release and increasing awareness for greener and sustainable routes for the extraction of natural products. Although conventional extraction techniques are cost-effective and easy to handle, it has certain limitations and also it demands a greater amount of raw material and solvent. Moreover, it requires higher energy leading to environmental burden. To increase the extraction efficiency with shrinking environmental burden and enhancement in energy conservation, sonication renders an alternative. The ultrasonic waves, in the extraction, have a frequency range of 20−100 kHz that is above the audible range of human beings.

Mechanism

Cavitation in the medium is the principle behind UAE. Waves when passing through medium cause cavitation in resultant to the pressure difference. The generation of alternating compression and rarefaction pressure results in microbubbles (Figure 1). Within the frequency of 20 – 100 kHz, microbubbles grow during the rarefaction cycle. The bubbles could be of two types inertial and non-inertial [17]. Both bubble types induce changes into the system. Bubble will eventually grow till it reaches an unstable phase when it implodes, and assists in the extraction of essential oil by rupturing or damaging the cell. The implosion results in a rise in local temperature which accelerates the extraction process (Figure 1). Micro-streaming and stress generation on the solid surface aid the faster extraction process.

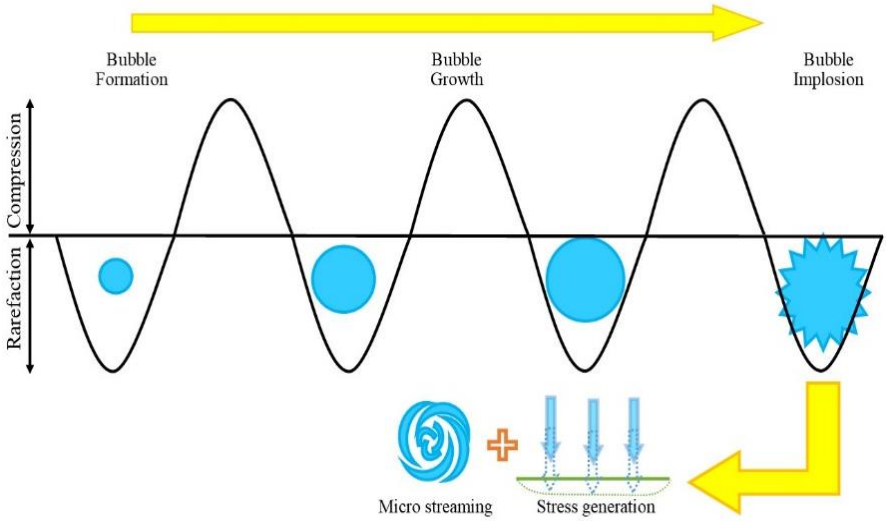

Figure 1. Relation between acoustic wave and bubble growth.

This phenomenon was observed in the extraction of essential oil from dry Chinese medicine by Feng et al. [18]. Thus, the incorporation of ultrasound is known to reduce the extraction time, save cost to the

environment and solvent consumption. Driven by these advantages, the ultrasound-induced processes have been termed as clean and green technologies [19]. Various attempts have been made to isolate essential oil using sonication which are reported in Table 1.

Incorporation of sonication in extraction, six types of physical damage have been observed by researchers viz. fragmentation, erosion, sono capillary, sonoporation, local shear stress and detexturation [19]. Due to inter-particle collisions and shock waves generated by ultrasound, fragmentation of raw material was observed during the extraction of essential oil from *Cymbopogon martini* [20, 21]. In certain cases, localize effect was observed wherein trichomes on the surface of leaves was impacted which is known as erosion. This type of physical damage enhances the diffusion of the solvent into the plant material leading to faster extraction [19] which is in validation with the extraction of essential oil from Boldo leaves [22]. Petigny et al. have observed that trichomes under the cavitation of bubbles, were damaged which enhanced the penetration capacity of water inside the leaf. Localize physical damage because of increase in depth and velocity of penetration of solvent resulting into canal type structure is defined as sono-capillary effect [23]. A similar effect was observed when rosemary leaves were subjected to ultrasound for the extraction of metabolites [24]. It was observed that after 40 min of treatment, leaf was destructed which has resulted into the dispersion of solvent. Sonic waves have been found very prevalent in natural product extraction where the pore like damage was observed which increased the permeability of solvent resulting into reduced extraction time [24]. The extraction of citronella oil using sono-hydrodistillation has led to pore formation on the surface of leaf as observed during morphological analysis. This phenomenon has resulted into reduced extraction time and carbon footprints [25]. Ultrasonic waves have ability to produce shear force within the bulk liquid and nearby liquid to the solid surface. This can lead to a rise in temperature causing the damage in glands for the extraction of natural products. The micro-streaming due to shear force affects the texture of plant material which is known as detexturation [19].

EFFECT OF PARAMETERS

The ultrasound assisted extraction of essential oil can be influenced by many factors namely solid loading, extraction time, ultrasound amplitude and power. In addition to this, ultrasonic transducers can be operated in continuous and pulsed mode. Pulse mode is operated in an on – off cycle which can save power, decrease the chances of degradation of components and erosion of probe [26]. Hashemi et al. found that no significant change in yield of oil was observed when ultrasound system was operated in pulse or continuous mode; however, the composition of oil got varied. More components were identified when extraction performed in pulsed sonication than continuous [26].

Solid Loading

Solid loading is the amount of plant material fed to the system for the extraction of essential oil. Due to mechanical form of sonic waves, solid loading has a critical impact on the process. The increase in solid loading would reduced the ultrasonic wave energy per particle therefore after a certain value, the extraction efficiency would get reduce. However, there was no evidence observed for essential oil extraction where the effect of varied solid loading would have been measured. A higher solid loading would also not allow the suitable cavitation of bubble which could lead to agitation of slurry formation as seen by Thakker et al. [21]. Ping et al. observed the increment in extraction rate and yield of cinnamon oil, however after a certain level, both have started decreasing. It has been perceived that increased solvent volume might have absorbed ultrasound energy resulting in a reduction in extraction temperature and yield of oil [27]. Similarly, Solanki et al. varied solid loading from 9 to 17 g wherein the negative effect of rise in solid loading was observed [25]. Increased solid loading has decreased the ultrasound energy per particle leading to reduced yield of oil. Sicaire et al. varied the solid to liquid ratio from 1/7 to 1/25. It was observed

that solid loading has a significant impact on the extraction from oleaginous seeds. The results are presented in Table 1.

Extraction Time

The physical alteration in the plant morphology increases the mass diffusion leading to the better extraction of essential oil. Li et al. studied the effect of extraction time on the yield of oil where the process was carried out from 10 to 60 min. The yield of oil increased till 30 min and later decreased which would be due to loss of volatile matter [27]. Similarly, sonication time was varied from 15 – 90 min during optimization using Box – Behnken design based response surface methodology and 52.5 min was obtained as optimum time while treating lavender flower. The extraction time was found to have a significant positive impact [28]. The extraction from traditional Chinese medicine was performed for 6 – 12 min by Feng et al. wherein the best quality of outcome was observed at 10 min duration. Due to resultant irradiation, this was considered to be the most suitable time for the outflow of volatile oil [18]. For extraction of palmarosa oil, the sonication time was varied from 4 – 20 min. The ANOVA study verified the extraction time to be a significant parameter for extraction. 16 min was considered as the best suitable condition to achieve the maximum yield of oil [21]. Similar fact was observed when citronella oil was extracted from Java citronella using sonication combined with conventional hydrodistillation in which the time was varied from 5 – 21 min. ANOVA study demonstrated that extraction time alone and in interaction with pulsation time has significantly influenced the extraction, and 21 min of extraction time was obtained as an optimum condition. The extraction of essential oil from *Kaempferia galangal* L. was performed in the time duration from 10 to 60 min with the incorporation of subcritical water system. The yield increased to 20 min, then no apparent change was observed [29]. An isolation of essential oil from cloves from 30 to 60 min was performed by Tekin et al. in the sonication bath. It was observed that the time period in combination with extraction temperature

had a significant impact on the yield of oil [30]. A rise in temperature increases the vapor pressure of solvent while viscosity and surface tension between liquids reduces which might increase the efficacy of process at a higher temperature. When extraction using sonication was compared with the conventional process, the time of extraction of essential oil from rape seed oil was found to be reduced to half. The complete extraction of oil was achieved in 60 min instead of 120 min [31]. When sonication was combined with conventional Clevenger extraction unit, the extraction rate was found to increasetill 20 min and later for 80 min no change in rate was observed [32].

Ultrasound Amplitude

Ultrasound amplitude allows the transducer to operate at the desired level which emits the limited waves. Amplitude is relevant to the frequency of ultrasound thus, at a higher frequency, lower is the amplitude. Increasing amplitude might lead to agitation of the solvent rather than the generation of cavitation bubbles [19]. The study on the varying amplitude was not noticed but it could have a significant impact on the process. Similarly, ultrasonic power also has a major influence on the process, however, it was not varied by various authors to study its effect. It has been observed that ultrasonic probe with high power is being used for the extraction of essential oil in the range of 100 – 500 W [18, 29, 33]. Solanki et al. have studied the effect of amplitude on the extraction of citronella oil, thus varied from 30 – 60% of total amplitude. It was found that increased amplitude led to a change in the pressure over the surface of the plant material. This in result has changed the magnitude of bubble collapse and cavitation mechanism [25].

Table 1. Ultrasound assisted extraction of EO

Sr. No.	Common name	Botanical name	Mode of Extraction	Optimum (Range) parameter	Yield (%, w/w)	Remarks	Ref.
1	Dahurian angelica flower	*Angelica dahurica*	Ultrasonic probe + microwave	T: 10 (8 – 12) min, P: 400 (0 – 800) W	-	Composition analysis	[18]
2	Cinnamon bark	*Cinnamomum cassia*	Ultrasonic bath	T: 40 (10 – 60) min, S/L: 6/60 (6/30 – 6/90), P: 165 W	14.37	Antibacterial activity	[27]
3	Honey-suckles leaves	*Lonicera macranthoides*	Ultrasonic reactor	T: 30 min, S/L: 6/30, t: 45°C, F: 25 kHz	-	Volatile fraction analysis	[35]
			Microwave extraction	P: 200 W, S/L: 6/30, t: 10°C			
			Cold maceration	t: 720°C			
			Hydrodistillation	T: 360 min, S/L: 1/10, t: 60°C			
			Soxhlet extraction				
4	Orange peel	*Citrus reticulate*	Ultrasound probe	T: 100 min, WR: 100 g, t: 110°C, F: 26 kHz	7.00	-	[32]
5	Lavender flower	*Lavandula angustifolia*	Ultrasonic microwave	T: 52.5 (15 – 90) min, P: 50 W, WR: 50 g, t: 40°C, F: 40 kHz	1.93	Biological activities	[28]
6	Black pepper seeds	*Piper nigrum*	Solvent free ultrasonic microwave assisted extraction	T: 7 min, P: 500 W, WR: 10 g, t: 100°C, F: 40 kHz	4.10	Composition analysis	[33]
			Ultrasound probe		3.40		
			Microwave assisted extraction	T: 7 min, P: 100 W, t: 100°C	3.70		
7	Kencur leaves	*Kaempferia galangal*	Ultrasound enhanced subcritical water extraction	T: 20 min, P: 250 W, SL: 100 g, t: 120°C, F: 20 kHz	2.45	Composition analysis Antioxidant activity	[29]
			Steam distillation	T: 210 min, SL: 100 g	2.00		
			Subcritical water extraction	T: 30 min, SL: 100 g, t: 120°C	2.43		
8	Clove buds	*Syzygium aromaticum*	Ultrasound assisted extraction	T: 31 (30 – 60) min, P: (40 – 100%), t: 30 (32 – 52) °C, F: 53 kHz	22.43	Optimization using RSM-CCD with antibacterial study	[30]
9	Rape seed	*Brassica napus*	Ultrasound assisted extraction	T: 40 min, t: 60 (15 – 55) °C, S/L: 1/15	21.20	Optimization using RSM-CCD	[31]
10	Orange peel		Ultrasound assisted extraction	T: 33 (15 – 35) min, S/L: 1/20	2.80		[26]

		Aloysia citriodora	Hydrodistillation	T: 153 min, t: 150°C	2.60	Antimicrobial study with composition analysis	
Sr. No.	**Common name**	**Botanical name**	**Mode of Extraction**	**Optimum (Range) parameter**	**Yield (%, w/w)**	**Remarks**	**Ref.**
11	Tunisian lemongrass leaves	*Cymbopogon flexuosus*	Ultrasound assisted extraction	T: 25 (10 – 30) min, P: 250 W, t: 50 (30 – 50) °C, S/L: 8/100, F: 40 kHz	3.09	Optimization using RSM-CCD	[34]
			Hydrodistillation	T: 300 min	1.85		
12	Palmarosa leaves	*Cymbopogon martinii*	Ultrasound assisted extraction	T: 16 (4 – 20) min, P: 60 (40 – 80) W, S/L: 2/65	1.89	Optimization using RSM-CCD	[21]
13	Citronella leaves	*Cymbopogon winterianus*	Sono-Hydrodistillation	PS: 25 (0.5 – 25) mm, SL: 5 (5 – 20) g, T: 21 (5 – 15) min, P: 100 (70 – 100) %, A: 70 (30 – 60) %, PR: 10:50 (10:50 – 40:20)	4.118	Optimization using RSM-CCD	[25]
			Hydrodistillation	PS: 25 mm, SL: 15 g, P: 500 W, T: 90 min.	2.33		
14	Cinnamon bark	*Cinnamomum zeylanicum L.*	Sono-Hydrodistillation	PS: 1000 (150 – 1000) µm, SL: 25 (10 – 30) g, T: 20 (5 – 20) min, P: 500 (300 – 500) W, A: 60 (30 – 60) %, PR: 27: 33 (10:50 – 40:20)	4.64	Optimization using RSM-BBD	[36]
			Hydrodistillation	SL: 20 g, P: 500 W	3.24		

PS: Particle size, A: Amplitude, PR: Particle size, T: Time, t: Temperature, F: Frequency (kHz), P: Power (W), RSM: Response surface methodology, CCD: Central composite design, BBD: Box-Behnken Design

Ultrasound Power

Acoustic power is not reported in many articles. It provides information for the applied energy to the system for the extraction of essential oil. In many cases, it is measured either in terms of frequency or power supplied directly. It has been noted that a higher power leads to major destruction on the plant material [18]. An increase in power causes more pressure on the surface of material leading to more abrasion and erosion [19]. Feng et al. have studied the effect of power on extraction wherein, the ultrasound power was varied from 0 to 800 W. Increasing power supply increased individual component peak because a higher power leads to the larger escape of components [18]. Similarly, Ping et al. operated the extraction of cinnamon oil at eight different levels like 99 to 330 W and observed that the rate of yield decreased with a rise in power [27]. Also, power was found to be significantly impacting the extraction. For extraction of cinnamon oil, the power of ultrasound would have either decomposed components of oil or volatile constituents might have escaped the system with a rise in temperature of the system. An increase in power in a few instances raised the local temperature of the system due to a higher abrasion effect [21]. Rise in temperature adversely impacts the yield and composition of the oil. Amine et al. have pretreated lemongrass before extraction using ultrasonic waves. The power was varied from 70 to 250 W in three levels and it was observed that at a higher power, the maximum amount of components was noted. The best condition for a better quality of oil was noted at a higher level of ultrasound power [34].

The study in ultrasound-assisted extraction mainly focused on the dissolution of oil into the solvent however, this may result into the presence of traces of solvent into the oil thereby compromising the oil quality.

ESSENTIAL OIL CHARACTERISTICS

Definite characteristics of essential oil are resultant of its chemical composition. The inclusion of ultrasound in the extraction process has

provided benefits in terms of extraction time, quantity of oil and carbon footprints. Researchers have not observed any change in the composition of essential oil. Since the properties of essential oil are because of its composition, no change in the characteristics of oil would be seen. Extraction from Angelica dahurica using ultrasound was found to be beneficial for obtaining higher components to maintain the medicinal characteristics [18]. Similarly, no change in antibacterial was observed when cinnamon oil was extracted using ultrasonic waves as reported in Table 1 [27]. The composition of palmarosa oil was improved when extracted using ultrasound wherein the geraniol composition was found to be higher [21]. Also, in the case of citronella oil extraction using sono-hydrodistillation, no change in composition was observed [25]. Essential oil from *Lavandula angustifolia* has resulted into the composition well in agreement with the literature [28] thus anti-pathogenic activity was also similar. Isolation of pepper oil using ultrasound was found to be better for the extraction of mono-terpenoids in comparison to the other techniques [33]. Similarly, Ma et al. observed an enhancement in the composition of oil thereby scavenging the effect of was stronger when extracted using ultrasound [29].

ULTRASOUND PRETREATMENT

Some of the researchers have applied ultrasonic waves as the pre-treatment before extraction of essential oil with conventional techniques. *Aloysia citriodora* leaves were pre-treated under sonication before hydrodistillation process. Ultrasonic treatment was given in pulsed and continuous form using ultrasound probe. Hashem et al. observed increment in yield of oil in comparison to the oil obtained without treatment. Also, the reduction in time of extraction was observed to a larger extent. It has been reported that the leave pretreated with ultrasonic waves might have physically altered the surface structure due to cavitation and micro-turbulence. Physical alteration leads to acceleration in the diffusion of oil from the plant core matrix to the solution. Ultrasound pre-treatment had a

clear effect of selective recovery wherein composition varied with the inclusion of pre-treatment steps [26].

ULTRASOUND-MICROWAVE ASSISTED EXTRACTION (UMAE)

To explore the benefits of microwave radiation and sonication, both were paired for extraction of EO [28, 33]. Black pepper, white pepper and lavender EOs were extracted utilizing such technique efficiently [28, 33]. Wang et al. have compared the individual microwave and ultrasound-assisted extraction with UMAE where the yield of white pepper has been increased by 10.8% and 20.6% with respect to MAE and UAE. Similarly, yield of EO from black pepper was increased by 29% from HD. Further, the quality of EO was improved [33] by increasing monoterpenes and sesquiterpenes compositions. Analogous to this, the lavender EO was isolated using ultrasound-microwave assisted technique in combination with enzyme and microwave which has provided the improved results [28].

COMPARISON AMONG VARIOUS TECHNIQUES

Apart from ultrasound assisted extraction, many other techniques like microwave assisted extraction, super/sub-critical fluid extraction, etc. were developed wherein varied form of energy or solvation power has been utilized. The experimental yield of essential oil mainly depends upon the maximum quantity of essential oil present and the part from which it is been extracted. Thereby, a comparison among various techniques with respect to common benchmark would give a better representation of the effect of each technique on the extraction of essential oil. The plant material as a benchmark has been selected as *Cymbopogon winterianus* grass which is commonly known as Java citronella grass.

The comparison was made among microwave assisted extraction, sono hydrodistillation and conventional hydrodistillation in terms of various

operating parameters, the yield of oil and energy conservation. It was observed that sonication has a major impact on the extraction yield than microwave energy. Both forms of energies have provided better response in comparison to conventional hydrodistillation. These techniques have also reduced the energy consumption thereby lesser carbon footprints were laid. Increasing awareness regarding natural products require betterment in the extraction technique such that it does not affect the earth's environment. Both MAE and SHD have proven to be a better alternative to the conventional process. However, the capital expenditure using ultrasound can be less compared to microwave radiation. The data represented in this section may change with the type of plant material, part of the plant utilized for extraction and its maturity pre- and post-harvesting.

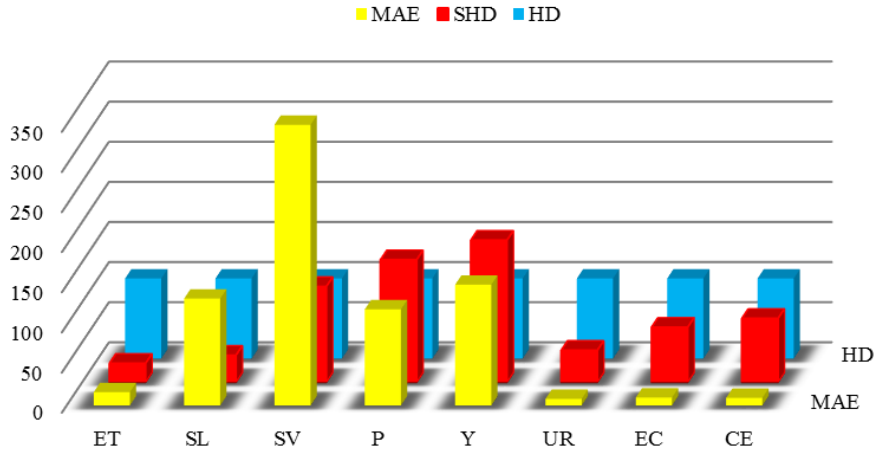

Figure 2. Comparison among various essential oil extraction techniques (ET: Extraction time, SL: Solid loading, SV: Solvent volume, P: Power, Y: Yield, UR: Utility requirement; EC: Energy consumption, CE: Carbon emission).

CONCLUSION

Essential oil is gaining importance in the replacement of the harmful chemical supplements for social betterment. This has led to a revolution in the consumption pattern of essential oil which requires a strong change in

the existing extraction technique. The change in technique should be aimed to enhance the yield of oil with reduced energy consumption and carbon footprint. The conventional hydrodistillation process is the basic, simplest and cost-effective technique for the extraction where thermal diffusion governs the process. Longer extraction period, high consumption of raw material as well as a solvent have been the major limitations.

For enhanced extraction of essential oil, novel extraction techniques were identified by varying heat sources or physical alterations such as ultrasound, microwave and ultrasound-microwave. Ultrasound-assisted extraction relies on the physical modifications in plant material which reduces the extraction time with increased yield of oil. Physical alteration in plant material plays a key role in enhanced extraction. The combination of microwave and ultrasound fulfils both the principles of heating and physical damage to overcome the drawbacks of each technique without affecting the quality and quantity of oil. Thus, sonication has the potential to alleviate the constraints posed by conventional methods.

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Chapter 3

PHYTOCHEMICAL CHARACTERIZATION AND EVALUATION OF ANTIBACTERIAL POTENTIAL AND MODULATOR OF ANTIBIOTIC RESISTANCE OF THE ESSENTIAL OIL OF *CAMPOMANESIA AUREA* O. BERG

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ABSTRACT

The resistance acquired by pathogenic microorganisms and the consequent inefficiency of antibiotics due to prolonged use are the main problems facing medicine today. Given this, numerous researches have been designed to look for new agents with antibacterial activity, including natural products. Thus, this study aimed to evaluate the antimicrobial activity of the essential oil of *Campomanesia aurea* O. Berg (EOCA) leaves, as well as to verify its antibiotic modulating potential. The essential oil was obtained by hydrodistillation in a Clevenger type device, with 0.17% content and the identification of the chemical compounds was done in a Mass Spectrometry Coupled Gas Chromatography (GC/MS) apparatus, where the majority compounds were obtained. The chemical compounds were made in a Mass Spectrometry Coupled Gas Chromatography (GC/MS) apparatus, where the major compounds were khusimol (11.7%) and epizizizone (8.7%). Antimicrobial activity was performed by the microdilution method to determine Minimum Inhibitory Concentration (MIC), with MICs of 101.59 and 256 μg/mL for *Staphylococcus aureus* 25923 and 10 respectively. The modulating effect of antibiotics was performed by combining the EOCA with the antibiotics Ampicillin, Gentamicin and Norfloxacin against the multidrug-resistant bacteria *Escherichia coli* 06, *Staphylococcus aureus* 10 and *Pseudomonas aeruginosa* 24, where an antagonistic effect with gentamicin front *S. aureus* was observed and synergism with norfloxacin and ampicillin antibiotics for *S. aureus* 10 and *P. aeruginosa* bacteria 24. There are still few studies analyzing the modulating activity of *C. aurea*, which is the first report of this activity.

Keywords: *Campomanesia aurea*, antibacterials, modulation, resistance

INTRODUCTION

Myrtaceae is among the 10 most diverse angiosperm botanical families in Brazil with around 1,031 species and 23 genera (Zappi et al., 2015; Sobral et al., 2015). The *Campomanesia* genus possesses 42 species, of which 32 are endemic, and is present in the Brazilian phytogeographic domains: Cerrado, Caatinga, Amazonia and Atlantic Forest, the latter being the richest with 36 recorded species (Sobral et al., 2015).

Campomanesia aurea O. Berg. is a fruit species native to the southern states of Brazil, popularly known as "guabiroba-do-campo," "goiabinha-do-mato" "araçazeiro-do-mato" or "guabiroba-araçá," occurring naturally from the state of São Paulo to Rio Grande do Sul (Lorenzi et al., 2006; Eme et al., 2018).

The *Campomanesia aurea* O. Berg. species flowers annually during the months of November and December with solitary flowers located in the leaf axils and at the base of the branches. Fruiting occurs between March and June, with the fruits being edible globular berries, with colors ranging from green to yellow according to the ripening stage (Stumpf, 2009; Molz, 2009).

Few reports addressing the chemical composition, antimicrobial and modulating activities of *C. aurea* exist in the literature; however, pharmacological studies evaluating the antimicrobial activity of the essential oil (Pacheco et al., 2014) and describing the physiognomy of the species (Marchiori and Santos, 2010) are being conducted.

Essential oils and their components are considered the most important antimicrobial agents present in plants and may also exhibit antioxidant and anti-inflammatory activity (Cowan, 1999). These naturally occurring chemical compounds with antimicrobial potential have become the targets of research seeking to find new substances capable of inhibiting the vital processes of common drug-resistant microorganisms. Moreover, such products may also act as resistance modulators (Gibbons, 2005), these being capable of further enhancing the expected mechanism of action effect when in association with antibiotics (Simões et al. 2009). Thus, this study aimed to evaluate the antimicrobial activity of the *C. aurea* essential oil, as well as to verify its potential antibiotic modulatory activity.

MATERIALS AND METHODS

Botanical Material Collection

Plant material collection for essential oil extraction was carried out in Campos Gerais, Vale do Ribeira and in the Paraná Coast, as well as in

Atalanta, Santa Catarina, where the collection of at least 10 species specimens was conducted.

The species was located, the coordinates ((latitude (S 25° 20.44'), longitude (W 49° 48.052') and altitude (1,063)) were registered and exsiccates were prepared in the field for botanical identification and photographic record. The exsiccates were transported to the Herbarium of the Spiritist Integrated Colleges, where they were herborized (Lawrence, 1973; IBGE, 1992) and listed in the collection under number HFIE 8.254.

Essential Oil Extraction

Essential oil extraction was performed by hydrodistillation for 2.5 hours in a Clevenger graduated apparatus with 50 g of dried leaves in 1L of distilled water (Wasicky, 1963). An electric dryer (FANEM - Mod. 320 SE) with air circulation at 40ºC for 24 hours was used to dry the leaves. 20g samples were collected in triplicates to determine the moisture content of the fresh leaves at the time of extraction, where these were submitted to drying in an electric dryer (FANEM - Mod. 320 SE) with air circulation at 65ºC until reaching a constant weight. After extraction, the sample was collected using precision pipettes and placed in a freezer where they remained until analysis. To determine the dry essential oil content, the total essential oil mass produced with respect to the dry botanical material mass used in the extraction was measured.

Essential Oil Chemical Composition Determination

The chemical constituents were identified by gas chromatography coupled to mass spectrometry (GC/MS). The essential oils were diluted in dichloromethane to a 1% ratio and 1.0 µL of the solution was injected with a 1:20 split flow rate in an Agilent 6890 (Palo Alto, CA) chromatograph coupled to an Agilent 5973N selective mass detector. The injector was kept at 250°C. Constituent separation was obtained using a HP-5MS capillary

column (5%-phenyl-95%-dimethylpolysiloxane, 30 m x 0.25 mm x 0.25 μm) and helium as the carrier gas (1.0 mL min^{-1}). The oven temperature was set from 60 to 240°C at a rate of 3°C min^{-1}. The mass detector was operated in the electronic ionization mode (70 eV) at a rate of 3.15 scans s^{-1} and a 40 to 450 u mass range. The transfer line was maintained at 260ºC, the ion source at 230ºC and the analyzer (quadrupole) at 150ºC.

The diluted sample was injected into an Agilent 7890A chromatograph equipped with a flame ionization detector (FID) operated at 280°C for quantification. The same column and analytical conditions described above were employed, except for the carrier gas used, which was in this case hydrogen at a flow rate of 1.5 mL min^{-1}. The percentage composition was obtained by the electronic integration of the FID signal by dividing the area of each component by the total area (area %).

Identification of the chemical constituents was obtained by comparing their mass spectra with those of spectral libraries (Mclafferty, Stauffer, 1994; NIST, 2016) as well as by their linear retention indices calculated from the injection of a homologous hydrocarbon series (C7-C26) and compared with literature data (Adams, 2007).

MICROBIAL STRAINS

The microorganisms used in the assays were obtained from the Laboratory of Microbiology and Molecular Biology (LMBM) of the Regional University of Cariri (URCA). Using standard *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 25923 bacterial strains, as well as multidrug resistant *Escherichia coli* 06, *Staphylococcus aureus* 10 and *Pseudomonas aeruginosa* 24 strains. Microorganism suspensions were produced from these samples in tubes containing 3 mL of sterile 0.9% sodium chloride. The suspensions were then shaken with the aid of a Vortex® and their turbidity was compared and adjusted to the McFarland scale (0.5-1.5x10^8 cells.mL^{-1}) (NCCLS, 2003).

Substance Preparation

10 mg of the essential oil were weighed in an Eppendorf and diluted with 9.765 mL of distilled water making up a 1024 µg/mL solution which was used in the MIC and modulation tests.

Culture Media

For the antibacterial evaluation assays Heart Infusion Agar (HIA solid media) and Brain Heart Infusion (10% BHI liquid media), prepared according to the manufacturer's instructions, were used. The media were solubilized with distilled water and autoclaved at 121°C for 15 minutes.

Drugs and Reagents

The antibiotics Gentamicin, Ampicillin and Norfloxacin were the reference drugs used in the tests, all prepared at an initial concentration of 1024 µg/mL, dissolved in sterile water to obtain the desired concentrations and decrease toxicity.

Minimum Inhibitory Concentration (MIC) Determination

The tests were performed using the broth microdilution technique with 96-well plates in triplicates. Bacterial suspensions were prepared in eppendorfs®, each containing 1,350 µL of 10% BHI and 150 µL of the bacterial inoculum (corresponding to 10% of the solution). The plate was filled numerically by adding 100 µL of this solution to each well, followed by serial microdilution of 100 µL with the essential oil diluted in distilled water at the initial concentration of 1024 µg/mL, with the concentrations ranging from 512 to 0.5 µg/mL. The plates were then taken to an incubator for 24 hours at 37°C. Bacterial MIC was determined by adding 20 µL of

resazurin into each well and visually observing this after 1 hour. The MIC was investigated by observing the visible turbidity in each well and the resazurin-induced color change, noting the lowest concentration of the product capable of inhibiting bacterial growth (Sales et al. 2015). The last well in all the tests were not microdiluted since they were used as the bacterial growth control (Javadpour et al., 1996).

MODULATORY EFFECT OF THE COMPOUND ON CLINICAL ANTIBIOTIC ACTIVITY

The method proposed by Coutinho et al. (2008a) was used to verify the essential oil antibiotic modulatory action against the tested resistant strains, where the essential oil was tested at a sub-inhibitory concentration (MIC/8). Bacterial suspensions were prepared in eppendorfs® each containing approximately 1,162 µL of 10% BHI and 150 µL of the bacterial inoculum (corresponding to 10% of the solution) and the corresponding MIC/8 volume (µL) of the essential oil. Eppendorf® tubes containing 1,350 µL 10% BHI and 150 µL of the microorganism suspensions were prepared for the antibiotic control. The plate was filled numerically by adding 100 µL of the solution to each well, followed by a 100 µL addition of the antibiotic to the first well, proceeded by serial microdilutions at a 1:1 ratio up until the penultimate well and the plates were incubated for 24 hours at 37°C.

STATISTICAL ANALYSIS

The data was analyzed by a two-way ANOVA followed by Bonferroni's post hoc test where $p < 0.05$ and $p < 0.0001$ were considered significant and $p > 0.05$ not significant.

RESULTS AND DISCUSSION

Gas chromatography coupled to mass spectrometry (gc/ms) analysis.

The presence of essential oils is an important characteristic for the Myrtaceae family with a mono and sesquiterpenes predominance existing (Soliman et al., 2016). These plant produced oils play a fundamental role in directing the use of chemical compounds for the development of herbal medicines and/or products directed towards pharmacology areas (Moresco, 2014).

Table 1. Essencial oil of *Campomanesia aurea* O. Berg

IR Calculated	Composition	%
1040	Limonene	1.6
1096	Terpinolene	0.6
1341	Delta-elemene	0.5
1394	Beta-elemene	0.9
1426	(E)-Caryophyllene	5.1
1457	Alpha-humulene	1.4
1464	Allo-aromadendreno	0.7
1490	Beta selinene	3.4
1500	Viridiflorene + bicyclogermacrene	3.8
1529	Trans-calamenene + delta-cadinene	3.0
1541	Alpha-cadinene + dracunculifoliol	1.3
1576	Spatulenol	1.6
1589	Caryophyllene oxide + globulol	6.0
1606	Guaiol + khusimona + rosifoliol	2.8
1635	1-epi-cubball	4.4
1652	Alpha-cadinol	5.0
1671	Epi-zizone	8.7
1732	Vetiselinenol	2.5
1756	Khusimol	11.7

In the OECA analysis a predominance of sesquiterpenes was observed, where this accounted for more than 89% of the oil's constitution, with only

two monoterpenes, limonene (1.6%) and terpinolene (0.6%), being identified. Species belonging to this genus generally present in their constitution a sesquiterpene predominance (Oliveira et al., 2016; Sá et al., 2018). The antimicrobial action of this species may be justified by the fact that sesquiterpenes have known protective functions against fungi and bacteria (Holetz et al., 2002).

The OECA yield obtained was of 0.17% of the dried samples. GC-MS analysis identified 19 chemical constituents with the major ones being khusimol (11.7%), epi-zizanone (8.7%) and caryophyllene oxide + globulol (6.0%), as shown in Table 1.

Do Amaral et al. (2018) reported a *C. aurea* essential oil content yield of 0.17% using 100 g of the species leaves when evaluating the content and chemical composition of Myrtaceae species in the Atlantic Forest of Paraná, where the compounds khusimol (11.7%) and epi-zizanone (8.7%) were identified as the major compounds for this species. These results suggest a uniformity between the chemical compounds these being characteristic of the species.

Antimicrobial Activity

Table 2 shows the minimum inhibitory concentration of the OECA, where for standard and multi resistant bacteria the OECA presented clinically irrelevant antibacterial activity against *P. aureus* ATCC 9027, *E. coli* ATCC 25922, *P. aureus* 24 and *E. coli* 06 with MIC values greater than or equal to 1024 μg/mL. Sá et al. (2018), obtained MIC values greater than 1000 μg/mL when using the *Campomanesia adamantium* leaf oil against *E. coli* and *P. aeruginosa* bacteria. Khusimol, a major *C. aurea* compound, obtained a MIC value of 1000 μg/mL in a study against *E. coli* (dos Santos et al., 2014).

MIC values of 101.59 and 256 μg/mL were obtained for the standard and multi resistant gram-positive bacteria *S. aureus* ATCC 25923 and *S. aureus* 10, respectively, where these results corroborate with the study by

Dos Santos et al. (2017), who evaluated the antibacterial activity of the *Campomanesia guazumifolia* (Cambess.) O. Berg. oil which obtained a MIC of 15 ± 0.1 μg/mL against *S. aureus*.

Antibiotic Modulatory Activity

Research on the combination of drugs and natural products has been a widely used technique in an attempt to reverse antibiotic resistance by increasing antibiotic activity or reversing resistance (Coutinho et al., 2015a; Coutinho et al., 2008b).

According to the results shown in Figure 1, the association of the OECA with gentamicin was antagonistic against *S. aureus* 10, i.e., this association increases the antibiotic's MIC from 64 to 406.4 μg/mL. The antagonistic effect of substances used in combination with antibiotics may be explained by the compounds binding to the drug target point or an antibiotic chelation mechanism, thereby reducing the effect spectrum of the drug (Costa et al. 2019; Oliveira et al., 2017; Coutinho et al., 2015b).

The OECA presented synergism when combined with norfloxacin and ampicillin against *S. aureus* reducing the MIC from 256 μg/mL to 203.187 μg/mL and 322.54 μg/mL to 128 μg/mL, respectively. This effect may be due to the cell wall structure of Gram-positive bacteria which are generally more sensitive to the action of antimicrobial agents (Zago et al., 2009), thus, the interaction between the essential oil and the drugs potentiated their antibacterial action.

All antibiotic modulation results were irrelevant against the Gram-negative *E. coli* 06 bacteria, which may be explained by the fact that Gram-negative cell walls reduce molecule absorption and movement into cells through porin channels (Tortora 2012).

Table 2. Minimum inhibitory concentration (MIC) values (µg/mL) Essencial oil of *Campomanesia aurea* O. Berg

Substance	Bacteria					
	S. A. ATCC 25923	P. A. ATCC 9027	E. C. ATCC 25922	SA 10	PA 24	EC 06
EOCA	101.59	≥1024	≥1024	256	≥1024	≥1024

ATCC - Standard strain; S. A. - *Staphylococcus aureus*; E. C. - *Escherichia coli*; P. A. - *Pseudomonas aeruginosa*; EOCA - Essencial oil of *Campomanesia aurea*.

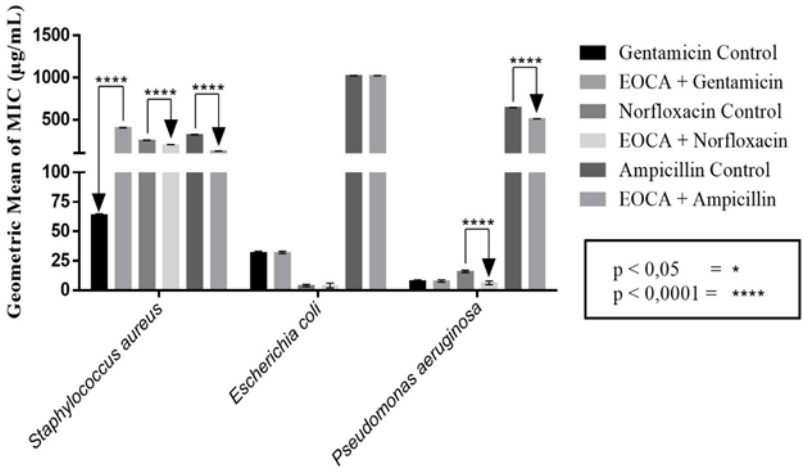

Figure 1. Modulatory effect of essential oil from *Campomanesia aurea* O. Berg in the antibiotic activity of Norfloxacin, Ampicillin and Gentamicin against multidrug resistant strains of *S. aureus* 10 (SA10), *E. coli* 06 (EC 06) and *P. aeruginosa* 24 (PA 24).

Synergism was observed against *Pseudomonas aeruginosa* 24 when the OECA was combined with norfloxacin and ampicillin, with their MIC values being reduced from 16 µg/mL to 6.3496 µg/mL and 645.08 µg/mL to 512 µg/mL, respectively, while an irrelevant result was observed when the OECA was associated with gentamicin. The resistance of Gram-negative bacteria such as *P. aeruginosa* is common considering this class of bacteria may present a permeability barrier due to the chemical composition of their

cell wall (Bassam 2004), thus becoming insensitive to essential oil compounds (Wang et al., 2012; Obidi et al., 2013; Ayoola et al., 2008).

CONCLUSION

A predominance of sesquiterpenes in the chemical composition of the *Campomonesia aurea* essential oil exists, which may be responsible for its antimicrobial activity.

The *C. aurea* essential oil when associated with antimicrobial drugs presented mostly synergistic events, potentiating their action against the tested bacteria.

Studies analyzing the modulatory activity of *C. aurea* are still scarce, with this study being the first to report on this activity. Studies with this approach should be encouraged to better understand the mechanism of action of this essential oil.

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Chapter 4

PHYTOCHEMICAL CHARACTERIZATION AND EVALUATION OF THE *EUPATORIUM INTERMEDIUN* DC. (ASTERACEAE) ESSENTIAL OIL MODULATORY AND ANTIBACTERIAL ACTIVITIES

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ABSTRACT

Lorem Studies with *E. intermedium*, the focus of this study, are still scarce, especially those addressing the chemical composition of its oils and extracts, or its biological, toxicological and pharmacological activities. This study aims to investigate the chemical composition of compounds from the E. intermedium leaf essential oil, in addition to its modulatory and antibacterial action. (E)-caryophyllene, α-humulene, germacrene D, bicyclogermacrene, spathulenol, elemicin and caryophyllene oxide were the main constituents found in the EIEO, with germacrene D being the most expressive in terms of percentage (17.2%). The EIEO demonstrated significant antibacterial effect only against *S. aureus* in the MIC assays, demonstrating a modulatory effect against *S. aureus* (S.A.10) and *E. coli* (E.C.06) in a synergistic manner. However, the oil acted as antagonist for *P. aeruginosa* (P.A.24), blocking the effect of ampicillin and norfloxacin. These results contribute to the discovery of new antibiotics from natural products, especially in the treatment of multiresistant bacteria. The essential oil was obtained by hydrodistillation using a Clevenger extractor. Gas chromatography coupled to mass spectrometry (GC-MS) was used for phytochemical analysis. The antibacterial and modulatory activity assays were performed using the broth microdilution method to establish the Minimal Inhibitory Concentration (MIC) as well as the oil's potentiating effect for the chosen antibiotics against three multiresistant bacterial strains: *Staphylococcus aureus*; *Escherichia coli* and *Pseudomonas aeruginosa*.

INTRODUCTION

The use of plants with medicinal purposes is an antique practice among human communities that remains embedded in current times (Oliveira et al., 2012). This fact has caught the interest of the scientific community, which have the development of new drugs using popular knowledge in mind, given their knowledge surrounding the possible biological properties of natural compounds (Johann et al., 2012).

Brazil is a country possessing one of the biggest biodiversities in the world, where roughly 43,496 plant species are described and which have an endemic rate of 56% of the world's species, placing Brazil in first place from the 17 megadiverse countries ranking (Forzza et al., 2012).

Eupatorium intermedium is a plant native to southern Brazil belonging to the Asteraceae family, known for its species wealthy (Di Stasi; Hiruma-Lima, 2002).

This species' characteristics are the following: branching shrub with 1.0 to 1.5 m in height, densely leafy up until inflorescences, which are composed of white flowers. While the species is typical of southern Brazil, it can establish growth from Minas Gerais, to Rio Grande do Sul and Uruguay. Its flowering period occurs from November to May and the species is usually found in dirty hillside fields, slopes and hills, these being areas with high solar luminosity incidence (Souza et al., 2007).

Given the *Eupatorium* genus and their medicinal usage, species from this genus have been reported in the literature as sources involved with the treatment of nausea, diarrhea, diphtheria and skin diseases. Other pharmacological effects have also been reported, such as: hepatoprotective, antipyretic, anticancer, diuretic, antispasmodic, cardiac stimulant, hemostatic, expectorant, antisyphilitic, antimalarial, antihemorrhagic (Hensel et al., 2011; Yu et al., 2017).

However, studies with *E. intermedium*, the focus of this study, are still scarce, especially those addressing the chemical composition of its oils and extracts, or its biological, toxicological and pharmacological activities. Therefore, the present study aimed to study the E. intermedium leaf essential oil chemical composition and to investigate its antibacterial and pharmacological modulatory action.

METHODS

Botanical Material Collection, Identification and Associated Authorization Ethical Aspects

The plant species was collected from the south of Brazil, in the state of Paraná on December 14, 2011, with 10 samples being obtained. After passing through the usual herborization techniques the botanical material

was sent for identification and registration at the Herbarium of the Municipal Botanical Museum of Curitiba (Herbário do Museu Botânico Municipal de Curitiba), the Herbarium of the Integrated Spiritist Faculties (Herbário das Faculdades Integradas Espírita) and the Royal Botanic Gardens Herbarium, Kew, England. Data referring to the geographic coordinates and the botany of the species are described in Table 1.

It should be noted that this study, given its access to Genetic Heritage, was regulated by the National Genetic Heritage and Associated Traditional Knowledge Management System (SisGen), with the registration number AAD6F13, thus complying with the expressed Law nº 13.133/2015 (Brazil, 2015).

Table 1. General data of the species *E. intermedium*

Scientific Name	**Family**	**N° Herbarium***	**Localization****		
			Latitude	Longitude	Altitude
Eupatorium intermedium	Asteraceae	HFIE 8.250	S 25º 20.461’	W049º48.046 ’	1.071

*Number of the specimen for the identified exsicata, as found in the Herbarium of the Municipal Botanical Museum (MBM) and Herbário das Faculdades Integras Espírita (HFIE) in Curitiba, PR. Herbarium Royal Botanic Gardens, Kew, England.

**Coordinates of collection of the species, this presents an average error of 15 m distance to the environment of the point.

Essential Oil Preparation and Phytochemical Analysis Procedures

The plant material was submitted to hydrodistillation in a Clevenger extractor to obtain the essential oil following collection and conditioning. In this equipment, adapted with a deep balloon, the botanical material was kept under heating at the temperature necessary to reach the boiling point.To quantify the percentage yield of the oil, the following calculation:

$$\text{Yield (\%)} = \frac{\text{Distilled essential oil volume (mL)}}{\text{Sample Mass (g)}}$$

For the chemical analysis, a GC-MS chromatographic procedure was performed in which oil samples were injected in 1% dichloromethane, using a separation ratio of 1:20, in an Agilent 6890 gas chromatograph (Palo Alto, CA) coupled to an Agilent 5973N selective mass detector. The injector temperature was maintained at 250°C. The chemical constituents were obtained by HP-5MS (5% phenyl - 95% - dimethylpolysiloxane, 30 m x 0.25 mm x 0.25 μm) capillary separation using helium as the carrier gas (1.0 mL min-1).

The apparatus was kept in an electronic ionization mode (70 eV) with the oven temperature programmed from 60 to 240°C at a rate of 3°C min, and a 3.15 scan s-1 rate and mass range of 40 to 450. The ion source was maintained at 230°C, the analyzer (quadruple) at 150°C and the transfer line at 260°.

For the quantification step, the sample was injected, after dilution, into the Agilent 7890ª chromatograph equipped with a flame ionization detector, known as a FID, which is responsible for obtaining the percentage compositions using the electronic integration of its signal operated at a temperature of 280°C. Hydrogen was used as the carrier gas with the same column described above, as well as the analytical standard, at 1.5 ml min1. Lastly, the mass spectra resulting from the entire process and linear retention indices, calculated after the injection of a homologous series of hydrocarbons (C_7–C_{26}), were analysed by comparison with the literature data.

Microbiological Assays

Strains and Drugs

For the experiments, three bacterial strains considered resistant to the antibiotics gentamicin, norfloxacin and ampicillin will be considered:

Staphylococcus aureus (S.A.10- S.A. ATCC 25923); *Pseudomonas aeruginosa* (P.A.24- ATCC 9027) and Escherichia coli (E.C.06- ATCC 25922). Bacterial cultures from the chosen strains were maintained at 4°C in Heart Infusion Agar (HIA, Difco), being cultured in Brain Heart Infusion (BHI, Difco) at 37°C prior to experimental assays.

Drugs

The antibiotics gentamicin, norfloxacin and ampicillin used herein were obtained from Sigma-Aldrich (USA). The preparations were made according to the Clinical and Laboratory Standards Institute Guidelines (CLSI) (NCCLS, 2003).

Minimal Inhibitory Concentration (MIC) Determination and Antibiotic Activity Modulation

The *E. intermedium* essential oil (EIEO) antimicrobial activity assays were performed using the broth microdilution method (Javadpour et al., 1996). According to Ostrosky et al., (2008), this technique is used to establish the MIC of the essential oil, considering standard *E. coli* and *S. aureus* strains, however, with some modifications, such as the addition of a third strain, *P. aeruginosa*. The final concentrations of the oil varied from 1024 to 0.5 μg/mL.

For the EIEO modulatory activity against antibiotic resistance, the MIC of the antibiotics were determined in the presence or absence of the EIEO at subinhibitory concentrations (128 μg/mL). Following the same procedure for the MIC assays, the plates were incubated for 24 hours at a temperature of 37°C, following microdilutions. All antibacterial assays were developed in triplicates (Coutinho; Cordeiro; Bringel, 2006; Javadpour et al., 1996, 32].

Statistical Analysis of Results

The data will be analyzed using a two-way ANOVA followed by Bonferroni's post hoc test, where $p < 0.05$ and $p < 0.0001$ are considered significant and $p > 0.05$ is not significant.

RESULTS AND DISCUSSION

EIEO Yield and Composition

The essential oil from *Eupatorium intermedium* leaves presented a yield of 1.5%. Results related to its phytochemical composition are shown in Table 2.

When comparing calculated IR with the literature, it was noted that 13 of the EIEO compounds were similar to the chemical composition of other species from the Eupatorium. *Eupatorium adenophorum* (Pandey et al., 2014), *Eupatorium africanum* (Babady-Bila et al., 2017), *Eupatorium ballotifolium* (Albuquerque et al., 2010), *Eupatorium cannabinum* (Pandey et al., 2014; Paolini; Costa; Bernardini, 2005) and *Eupatorium macrophyllum* (Maia et al., 2002) were evaluated as essential oil; *Eupatorium polystachyum* as a volatile oil (Souza et al., 2007). *Eupatorium intermedium* was also analysed however, this study contemplated extracts from the plant's flowers (Czaikoski et al., 2015).

The other three elements which were not found in Eupatorium studies had IR comparable with data from species belonging to the same genus in question, Asteraceae. γ-cadinene was found in the *Baccharis trinervis* (Chaverri; Cicció, 2017) and *Arnica montana* (Sugier et al., 2017) essential oils; elemicin in the *Artemisia dracunculus* (Muñoz- Acevedo; Kouznetsov; Stashenko, 2009) essential oil; and germacra-4(15), 5,10(14)-trien-1α-ol in the *Centaurea choulettiana* essential oil (Azzouzi et al., 2016), as well as in *Baccharis trinervis* (Chaverri; Cicció, 2017).

Table 2. Composition of the essential oil of *E. intermedium* (EOEI)

N°	Composto	%	IR[1]	IR[2]	Reference
1	β- bourbonene	3,3	1385	1372	Souza et al., 2007
				1386	Judzentiene, 2007
2	(E)- caryophyllene	14,9*	1421	1416	Czaikoski et al., 2015
				1427	Sugier et al., 2017
3	α- humulent	4,2*	1454	1452	Maia et al., 2002
				1455	Judzentiene, 2007
				1459	Babady-Bila et al., 2017
4	Allo- aromadendrene	2,0	1460	1458	Chaverri; Cicció, 2017
				1463	Maia et al., 2002
5	Germacrene D	17,2*	1482	1477	Paolini; Costa; Bernardini, 2005
				1480	Maia et al., 2002
6	Bicyclogermacrene	10,6*	1497	1493	Czaikoski et al., 2015
				1496	Maia et al., 2002
7	γ- cadinene	0,8	1513	1513	Chaverri; Cicció, 2017
				1526	Sugier et al., 2017
8	δ- cadinene	1,5	1523	1523	Judzentiene, 2007
				1527	Maia et al., 2002
9	Elemicin	4,3*	1563	1557	Muñoz- Acevedo; Kouznetsov; Stashenko, 2009
10	Spathulenol	6,5*	1574	1577	Albuquerque et al., 2010
11	Oxide of caryophyllene	4,4*	1580	1579	Czaikoski et al., 2015
				1580	Judzentiene, 2007
				1581	Maia et al., 2002; Pandey et al., 2014
12	Globulol	1,2	1588	1576	Paolini; Costa; Bernardini, 2005
				1588, 1644	Maia et al., 2002
13	Epi-α-muurolol	3,4	1642	1647	Budel et al., 2018
14	α-muurolol	3,9	1646	1645	Maia et al., 2002
15	α-cadinol	2,6	1654	1651	Czaikoski et al., 2015
				1653	Pandey et al., 2014
16	Germacra-4(15),5,10(14)-trien-1- α -ol	1,6	1684	1687	Chaverri; Cicció, 2017
				1689	Azzouzi et al., 2016

IR[1] = Retention rate calculated.

IR[2] = Index of retention of the literatureIndex of retention of the literature.

* = Compounds with higher percentage.

According to the percentages, the main EIEO constituents are: (E)-caryophyllene; α-humulene; germacrene D; bicyclogermacrene; spathulenol; elemicin and caryophyllene oxide. Four of these compounds are also characterized as majoritary in the *E. intermedium* flower phytochemistry, these being (E)-caryophyllene, germacrene D, bicyclogermacrene and caryophyllene oxide (Czaikoski et al., 2015).

Germacrene D, the most expressive element in the EOEI, was also observed by Paolini, Costa, Bernardini (2005), as the most relevant element in the *Eupatorium cannabinum subsp. corsicum* (L.) essential oil, which was also confirmed in the study by Judzentiene (2007), using the same species.

EIEO MIC and Antibiotic Activity Modulation

In the MIC determination assays, the results showed the EIEO presented significant action against the standard Gram-positive *S. aureus* ATCC 25923 sample, with a calculated value of 322.54 μg/mL (Table 3), however, for the other bacteria no responses (≥1024 μg/mL) were seen.

In extracts from *Eupatorium intermedium* flowers obtained by $scCO_2$, a similarity with the EIEO was observed. In tests with several strains, emphasizing here *S. aureus* and *E. coli*, an antibacterial effect was only observed for S.A. ATCC 25923 (Czaikoski et al., 2015).

Table 3. Minimum inhibitory concentration (MIC) values (μg/mL) the essential oil from *Eupatorium intermedium* DC.

Substance	**Bacteria**					
	S.A. ATCC 25923	P.A. ATCC 9027	E.C. ATCC 25922	S.A. 10	P.A. 24	E.C. 06
EOEI	322.54	≥1024	≥1024	≥1024	≥1024	≥1024

ATCC.- Standard strain; S.A.- Staphylococcus aureus; E.C.- Escherichia coli; P.A.- Pseudomonas aeruginosa; EOEI - Essencial oil of Eupatorium intermedium.

Considering other *Eupatorium* species, *Eupatorium adenophorum* Spreng aqueous and methanolic extracts studied by Chaudhary, Negi and Dahiya (2010), presented an antibacterial response against SA ATCC 25923, a result which was also observed with the essential oil of the same plant, according to data from, however Kurade (2010), using the SA MTCC 26 variation. The two studies also presented positive data against *E. coli*, an action reported by Sharma et al. (2013), for the *Eupatorium odoratum* Linn. essential oil which, however, was not obtained with application of the EIEO.

This may have occurred due to the complexity of Gram-negative bacteria walls (*E. coli* and *P. aeruginosa*), which are coated by an outer membrane, which makes its surface strongly hydrophilic, thus preventing the action of hydrophobic compounds (Czaikoski et al., 2015).

Another explanation is that differences may exist for the antibacterial effect of oils from the same genus, due to circumstances such as chemotype variability, vegetative cycle and even by the process of obtaining the oils (Sharma et al., 2013).

On the other hand, the EIEO was seen to generally potentiate the action of the antibiotics against *S. aureus* (S.A.10) and *E. coli* (E.C.06) bacteria in a synergistic manner in the modulation assays. However, for *P. aeruginosa* (P.A.24) the oil acted as an antagonist, blocking the effect of ampicillin and norfloxacin. As in the MIC assay results, the EIEO presented more relevant results against *S. aureus* in the modulation assays (Figure 1).

Although studies on the antibacterial effects of species from genus *Eupatorium* exist, no studies focusing on the modulatory activity of the species were found in the literature.

However, for being an essential oil, this type of botanical product possesses important characteristics capable of explaining their mechanisms of antibacterial action. Hydrophobicity is an aspect that provides the oil with the ability to alter lipids in the bacterial cell membrane, thus making it more permeable to ions and antibiotic molecules causing cell death (Fernandes et al., 2014).

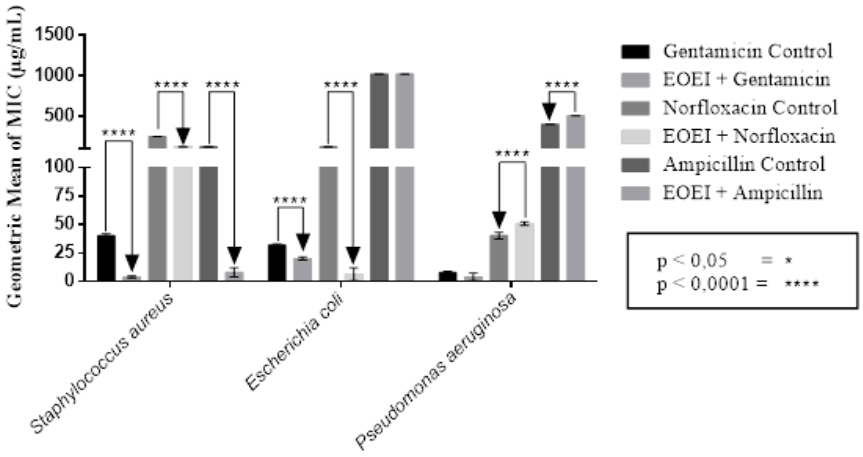

Figure 1. Modulatory effect of essential oil from *Eupatorium intermedium* DC. in the antibiotic activity of Norfloxacin, Ampicillin and Gentamicin against multidrug resistant strains of S. aureus 10 (SA10), E. coli 06 (EC06) and P. aeruginosa 24 (PA24).

According to Pelissari, Pietro, Moreira (2010), essential oils can also impair lipid-protein interactions by accessing the proteins of cytoplasmic membranes, such as ATPases, triggering accumulation in the lipid bilayer. In addition, direct interaction of lipophilic and hydrophilic compounds can occur, increasing bacterial destruction effectiveness.

While germacrene D is the compound with the highest expressivity, it is probably not the antibacterial action mediator since previously carried out tests revealed its inactivity for the following pathogens: *Bacillus subtilis, B. cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Micrococcus roseus, M. luteus, Candida albicans, C. dubliniensis, C. krusei* and *Saccharomyces cerevisiae* with concentrations up to 5 mg/mL (Oliveira et al., 2012a).

However, the majority of phytochemicals found in the EIEO were terpene compounds, with its main constituents belonging to the sesquiterpene class. Spathulenol, for example, obtained by the oxidative cyclization of bicyclogermacrene, shows high hydrophobicity, due to its ability to cross the plasma membrane, causing K^+ ions from bacterial cells

to be extravasated with the efficacy of this action being stronger in *S. aureus* (Cazella et al., 2019).

Other elements may also be associated with antibacterial and modulating effects. According to Murari et al. (2008), α-cadinol presents bactericidal activity, such as a protein denaturant, solvent or dehydrating agent, thus improving and facilitating the efficacy of synthetic antibiotics, despite not presenting an expressive percentage in relation to other EIEO compounds.

Costa et al. (2009), explains that essential oil phytocompounds can affect bacterial structures in different ways. Thus, studying these elements in an isolated manner or in synchrony between them, is a relevant way to acquire more precise knowledge regarding their antibacterial activity.

CONCLUSION

Results from this work have demonstrated the *Eupatorium intermedium* essential oil presents an effective antibacterial potential against the *S. aureus* strain. This data serves for future studies addressing its performance in other Gram-positive bacteria.

The EOEI acted as a potentiator for antibiotic action against the three bacteria, varying according to the antibiotic type and strain. These results can be considered unprecedented and innovative for the scientific community, since there is still no data on the modulation of *Eupatorium* species in the literature.

The importance and relevance of studies focusing on a more detailed investigation of the mechanism(s) of action(s) of these phytochemical compounds with respect to their modulatory and antibacterial activity are also highlighted.

Lastly, the findings from this study are promising with respect to the contribution to the discovery of new antibiotics derived from natural products, especially for the treatment of multiresistant bacteria.

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Chapter 5

ROLE OF SANDALWOOD OIL AND ALPHA-SANTALOL IN THE PREVENTION AND DEVELOPMENT OF HUMAN MALIGNANCIES

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ABSTRACT

Different cultures from around the world have been using natural products for many centuries, not only to promote human health but also prevent the development of various chronic diseases. A large body of evidence clearly demonstrates the medicinal value of a number of phytochemicals derived from these natural products. These phytochemicals have been extensively investigated for their role in disease modulation and progression. One such natural product is sandalwood oil

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that has been used as a traditional medicine for treating different ailments including cancer. Sandalwood oil constitutes alpha-santalol, a sesquiterpene that has been studied for its health benefits and its ability to modulate different signaling pathways involved in the development of a malignancy. For example, the antitumor and cancer preventive properties of alpha-santalol have been shown to involve cell death induction through apoptosis and cell cycle arrest in various cancer models. Alpha-santalol also decreased inflammatory markers associated with neurodegenerative and skin disorders. This chapter summarizes the biological aspects attributed to the medicinal benefits of sandalwood oil and alpha-santalol against various ailments with relevant clinical evidence.

ABBREVIATIONS

Cdc25B	cell division cycle 25B
CDKs	cyclin dependent kinases
COX-2	cyclooxygenase-2
DHA	docosahexaenoic acid
DMBA	7,12-dimethylbenz(a)anthracene
EGFR	epidermal growth factor receptor
EPA	eicosapentaenoic acid
ER	estrogen receptor
EISO	East Indian Sandalwood Oil
PUFAs	polyunsaturated fatty acids
GST	glutathione S-transferase
HNSCC	head and neck squamous cell carcinoma
HUVECs	human umbilical vein endothelial cells
IAP	inhibitor of apoptosis
i.p.	Intraperitoneal
ODC	ornithine decarboxylase
PARP	poly ADP-ribose polymerase
PDE	phosphodiesterase
PCNA	proliferating cell nuclear antigen
ROS	reactive oxygen species
SKN-1	endocrine Signaling Pathway

SWO	sandalwood oil
TPA	12-O-tetradecanoyl-phorbol-13-acetate
VEGFR2	VEGF receptor 2
6-OHDA	6-hydroxydopamine

INTRODUCTION

The quest for agents that can not only prevent/treat a chronic disease but also limit the occurrence of adverse effects has led to the use of plant-derived compounds and essential oils for their anti-inflammatory, anti-proliferative and anti-hyperglycemic effects. Sandalwood oil (SWO), an essential oil, extracted from the heartwood of *santalum album* tree is comprised of several structurally related compounds. Alpha-santalol (Figure 1), present in highest levels, has gained momentum in the scientific community. Traditionally, SWO has been used in different cultures for its medicinal value. Accumulating evidence suggests that SWO and its components exhibit various biological properties essential in preventing the development of chronic and infectious diseases. Studies, including those from our group, have shown the growth suppressive properties of alpha-santalol against a wide range of human tumor cell lines. In this chapter we highlight studies on the cancer preventive properties of alpha-santalol, including results from cell culture and animal models. Studies focusing on the anti-inflammatory and neurological effects, as well as other applications, are also reviewed in great detail.

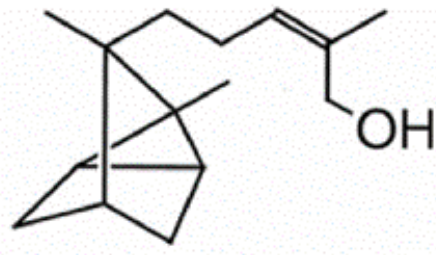

Figure 1. Structure of alpha-santalol.

In addition, we also discuss clinical trials examining the toxicity and efficacy of SWO. Despite the evidence pertinent to the medicinal value of these natural agents, our knowledge regarding the full spectrum of their pharmacological activities is limited. Additional *in vitro* and *in vivo* studies of the potential preventive effects of these agents are warranted. This chapter updates our previous review of medicinal properties of alpha-santalol (Bommareddy, 2017).

ANTI-TUMOR EFFECTS

Based on the skin protective benefits of sandalwood preparations, exploring their potential benefits against prevention of various skin ailments including non-melanoma skin cancer was logical. To that end, SWO and its components, including alpha-santalol, have been extensively studied for their antitumor benefits employing various cancer models. A large body of literature from *in vitro* and *in vivo* studies report the cancer preventive properties of SWO and alpha-santalol. The unique characteristics associated with these natural agents is their ability to target malignant cells and have little to no toxicity towards normal cells. The anticancer properties of sandalwood against various cancer models was recently reviewed (Santha and Dwivedi 2015). It is well documented that alpha-santalol exhibits cytotoxic effects against tumor growth through inhibition of angiogenesis and induction of cell-cycle arrest & apoptosis. A summary of the anti-tumor and chemopreventive properties of SWO and alpha-santalol is presented in Table 1.

Skin Cancer

The earlier studies focusing on the chemopreventive effects of sandalwood oil employing CD1 mice model for skin carcinogenesis revealed that topical application of SWO (5% w/v in acetone) reduced incidence of papilloma by 67% and tumor multiplicity by 96% (Dwivedi, 1997). In a

follow up study, SWO pre-treatment reduced papilloma incidence and multiplicity in a concentration and time-dependent manner (Dwivedi, 1999). Subsequent studies that focused on investigating the cancer preventive properties of alpha-santalol employing *in vivo* and *in vitro* models provided compelling evidence against skin cancer development. For example, treatment with alpha-santalol significantly prevented papilloma development during the promotion phase of DMBA and TPA carcinogenesis protocol in both CD-1 and SENCAR mice (Dwivedi et al., 2003, Dwivedi et al., 2005). In addition to chemically-induced skin carcinogenesis, alpha-santalol also had profound chemopreventive effects against UVB induced skin tumorigenesis in SKH-1 hairless mice under three different protocols (DMBA-initiated and UVB promoted; UVB-initiated and TPA-promoted and UVB initiated and UVB promoted). The treatment was most effective, with a 72% reduction in tumor multiplicity on UVB induced complete tumorigenesis (Dwivedi et al., 2006, Santha and Dwivedi, 2013). In the same study, the distribution analysis of alpha-santalol revealed that the chemopreventive effects of alpha-santalol are not due to its blocking ability; but possibly due to its systemic absorption into the skin (Dwivedi et al., 2006).

A subsequent study conducted in 2007 employing Female SKH-1 mice showed that 5% alpha-santalol in acetone was significantly more effective than vehicle or 2.5% alpha-santalol in reducing tumor development (Bommareddy et al., 2007). A follow-up study using the same mouse model which had a pretreatment of alpha-santalol one hour prior to UVB exposure resulted in a significant reduction in tumor incidence and multiplicity, and resulted in increased expression of apoptotic proteins (caspase-3 and 8) and tumor suppressor protein, p53 (Arasada et al. 2008).

Further studies revealed the mechanistic details involved with the anticarcinogenic action of alpha-santalol against UVB-induced photocarcinogenesis. It was identified that the alpha-santalol-mediated anticancer effects are associated with inhibition of inflammation, epidermal cell proliferation, cell cycle arrest and induction of apoptosis.

Table 1. Anti-tumor and chemopreventive properties

Lead Author	Year	Specimen	Cancer	Carcinogen	Treatment	Results
Dwivedi	1997	CD-1	Skin	7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) carcinogenesis	SWO (5% w/v) acetone	Decreased papilloma incidence by 67%, multiplicity by 96%, and TPA-induced ornithine decarboxylase (ODC) activity by 70%.
Dwivedi	1999	CD-1	Skin	DMBA initiated and TPA promoted carcinogenesis	SWO (1.25, 2.5, 3.75, 5% w/v) in acetone	SWO pre-treatment decreased the papilloma incidence and multiplicity in a concentration and time-dependent manner.
Dwivedi	2003	CD-1, SENCAR mice	Skin	DMBA initiated and TPA promoted carcinogenesis	alpha-santalol (5% w/v) acetone	Prevented papilloma development in both CD-1 and SENCAR mice, possibly by inhibiting TPA-induced ODC activity and DNA synthesis.
Dwivedi	2005	CD-1 mice	Skin	DMBA and TPA carcinogenesis	alpha-santalol (1.25 and 2.5%) in acetone	no significant difference in the effects of 1.25% and 2.5% alpha-santalol on tumor incidence, multiplicity, epidermal TPA-induced ODC activity, or DNA synthesis.
Dwivedi	2006	SKH-1 mice	Skin	UVB radiation	100 µl of α-santalol (5%, w/v, in acetone)	Topical application of alpha-santalol inhibited UVB-initiated and promoted skin tumor development.
Bommareddy	2007	SKH-1 mice	Skin	UVB radiation	2.5% & 5% alpha-santalol in acetone	Inhibited UVB-induced skin tumor development; higher concentration proved more effective.

Lead Author	Year	Specimen	Cancer	Carcinogen	Treatment	Results
Arasada	2008	SKH-1 mice	Skin	UVB radiation	alpha-santalol 1 hour prior to UVB exposure	Increased p53, caspase-3, & caspase-8 levels; reduced tumor incidence & multiplicity.
Matsuo	2012	N/A	HL-60 human promyelocytic leukemia cells	No	Seven alpha-santalane type sesquiterpenes	Exhibits tumor-selective cytotoxicity.
Bomma reddy	2012	N/A	Prostate; p53 deficient PC-3 & LNCaP cells	No	0-75 μM concentrations of alpha-santalol in DMSO	Induced apoptosis with activation of caspase-3 activity & PARP cleavage.
Chilamp alli	2013	SKH-1 mice	Skin	UVB radiation	topical administration of alpha-santalol (5 mg in 200 μL acetone); honokiol (30 μg in 200 μL acetone); alpha-santalol (5 mg in 100 μL acetone) and honokiol (30 μg in 100 μL acetone).	Induction of apoptosis
Santha	2013	SKH-1 mice	Skin	UVB radiation	Topical administration of alpha-santalol (10%, w/v in acetone)	Decreased expression of cyclins (A, B1, D1, D2) & Cdk1 (Cdc2), Cdk2, Cdk4 & Cdk6, upregulated expression of Cip1/p21, elevated levels of cleaved caspase-3 & PARP.
Santha	2013	N/A	Breast; p53 wild-type MCF-7 cells & p53 mutated MDA-MB-231	No	10-100 μM concentrations of alpha-santalol in DMSO	Inhibited cancer cell viability & proliferation by inducing G2/M cell cycle arrest & apoptosis; less toxic effect on normal breast epithelial cells.

Table 1. (Continued)

Lead Author	Year	Specimen	Cancer	Carcinogen	Treatment	Results
Saraswati	2013	Swiss Albino Mice (male nude)	Prostate; PC-3 or LNCaP cell s.c. xenograft	No	5-40 μM concentrations of alpha-santalol in DMSO	Reduced volume & weight of solid tumors, reduced the cell viability, & induced apoptosis.
Bommareddy	2015	N/A	Breast; p53 wild-type MCF-7 cells & p53 mutated MDA-MB-231 cells	No	20 μM and 40 μM concentrations of alpha-santalol	Down-regulation of total survivin protein. Suppression of survivin, not regulated through the PI3K–AKT pathway.
Lee	2015	Female athymic mice	Oral; Head and neck squamous cell carcinoma xenograft	No	Daily topical administration 50% EISO in DMSO: 5 μL days 0-2, 10 μL days 3-4, 20 μL days 7-11 & 14-18	Caused formation of multipolar mitotic spindles & disturbed microtubule polymerization, stopping the HNSCC in G2/M phases.
Dave	2017	Female Sprague-Dawley rats	Breast cancer	DMBA	Cream formulation of α-santalol (10% v/v in Dermabase® cream) or 10%, 25% v/v of alpha-santalol phospholipid microemulsions	Transdermal/Transpapillary delivery of α-santalol reduced tumor incidence and multiplicity in rat chemical carcinogenesis model of breast cancer.
Rao	2017	Humans	Skin	Ionizing radiation	Cream formulation of turmeric extract 16% *w/w*, Sandalwood Oil 0.5% *w/w* in a non-greasy base	Reduced radiation dermatitis in women receiving radiation therapy for breast cancer treatment.
Bommareddy	2018	N/A	Breast; MCF-7 cells MDA-MB-231 cells	No	20 μM and 40 μM concentrations of alpha-santalol	Reduced migratory potential and wound healing ability of breast cancer cells. Affected the localization of β-catenin from cytosol to nucleus in MDA-MB 231 cells.

N/A: Not Applicable.

Alpha-santalol pretreatment significantly inhibited UVB-induced epidermal hyperplasia and thickness of the epidermis, expression of proliferation and inflammation markers such as proliferating cell nuclear antigen (PCNA), Ki-67 and cyclooxygenase-2 (COX-2) (Santha and Dwivedi, 2013). A significant decrease in the expression of cyclins A, B1, D1 and D2 and cyclin-dependent kinases (Cdk)s Cdk1 (Cdc2), Cdk2, Cdk4 and Cdk6 and an upregulated expression of cyclin-dependent kinase (CDK) inhibitor Cip1/p21 were found in the alpha-santalol pretreated group, as well. Furthermore, an elevated level of cleaved caspase-3 and cleaved PARP were observed in the alpha-santalol treated group, indicative of proapoptotic activity (Santha and Dwivedi, 2013).

Studies from the same group employing non-melanoma and melanoma skin cancer cells demostrated G2/M phase cell cycle arrest upon alpha-santalol treatment in p53-mutated A431 human epidermoid carcinoma cells and p53 wild-type UACC-62 human melanoma cells. Knockdown of p21 in A431 cells or knockdown of both p21 and p53 in UACC-62 cells did not change G2/M phase arrest caused by alpha-santalol treatment (Zhang et al. 2010). Furthermore, studies performed employing combination approaches employing honokiol and magnolol revealed the chemopreventive potential of alpha-santalol against skin tumor development in SKH-1 mice when used together than individual compounds. Pretreatment of SKH-1 mice before exposing to UV radiation employing combinations of alpha-santalol with honokiol and magnolol significantly decreased tumor multiplicity up to 75% versus control, alpha-santalol, honokiol and magnolol alone (Chilampalli et al. 2013).

Oral Cancer

The earlier stages of oral cancer are most often treated with surgery, sometimes in combination with radiotherapy. However, this treatment frequently results in severe toxic side effects such as an increased exertion in speaking and/or swallowing. As the disease progresses, treatment of oral cancers includes chemotherapy, which presents undesired side effects that

may lead to a reduction in the quality of life. Unfortunately, advancements in disease diagnosis and management have not been substantial in HPV-negative head and neck squamous cell carcinoma (HNSCC) tumors in the past 30 years. Therefore, there is a need for the development of better treatments for HNSCC. Based on the results demonstrated, it is reasonable to conclude that alpha-santalol is a potential anticancer agent that can be used in the treatment of oral cancers (Lee et al. 2015). In the study, alpha-santalol was found to be cytotoxic against HNSCC lines, causing G2/M cell cycle arrest. Additionally, it was found that treatment with alpha-santalol caused formation of multipolar mitotic spindles analogous to those observed upon treatment of cells with compounds that disturb microtubule polymerization. Modeling studies propose that santalols can weakly bind to the colchicine site on tubulin. Therefore, santalols can directly interact with tubulin and inhibit the polymerization of microtubules, a prevalent mechanism of anticancer activity observed with established chemotherapeutic agents. However, alpha-santalol exhibits greatly reduced toxicity as compared to most other compounds having the property to interact directly with tubulin (Lee et al. 2015).

Breast Cancer

The group that demonstrated chemopreventive effects of SWO and alpha-santalol against skin cancer development also explored the mechanistic details behind alpha-santalol's antitumor efficacy in breast cancer cells (p53 wild-type MCF-7 cells as a model for estrogen receptor (ER)-positive and p53 mutated MDA-MB-231 cells as a model for ER-negative). Alpha-santalol inhibited cell viability and proliferation in a concentration and time-dependent manner in both breast cancer cell lines irrespective of their ER and/or p53 status. As expected, alpha-santalol exhibited less toxicity towards normal breast epithelial cell line, MCF-10A (Santha et al. 2013).

Treatment of breast cancer cells with alpha-santalol resulted in G2/M cell cycle arrest and apoptosis. Cell cycle arrest was associated with changes in the protein levels of BRCA1, Chk1, G2/M regulatory cyclins, cyclin dependent kinases (CDKs), cell division cycle 25B (Cdc25B), Cdc25C and Ser-216 phosphorylation of Cdc25C. An up-regulated expression of p21 along with reduced expression of mutated p53 was observed in MDA-MB-231 cells treated with alpha-santalol. On the contrary, alpha-santalol did not increase the expression of wild-type p53 and p21 in MCF-7 cells. In addition, alpha-santalol induced extrinsic and intrinsic pathways of apoptosis in both cells with activation of caspase-8, 9 and caused activation of the executioner caspase-6, 7 in MCF-7 cells and caspase-3, 6 in MDA-MB-231 cells along with PARP cleavage in both the cells (Santha et al. 2015).

As well as exploring the mechanistic details behind alpha-santalol's ability to induce cell-cycle arrest and apoptosis, more recent studies focusing on the mechanism by which alpha-santalol induces apoptosis using the same cultured breast cancer cell lines revealed that alpha-santalol treatment causes down-regulation of total survivin protein regardless of estrogen receptor (ER) and/or p53 status (Bommareddy et al. 2015). Overexpression of survivin is correlated with tumor recurrence and therapeutic resistance. Studies have shown that upon inhibition of the PI3K-AKT pathway, survivin levels are downregulated and tumor burden is reduced. In the study, involving the breast cancer cells, the association of the PI3K-AKT pathway and survivin down-regulation was explored. It was shown that alpha-santalol-mediated anticancer effects in breast cancer cells may be regulated, in part, through suppression of survivin which may occur not through the PI3K-AKT pathway but via a different pathway (Bommareddy et al. 2015). A follow-up study revealed that alpha-santalol-mediated growth suppression in breast cancer cells may be regulated through Wnt//β-catenin pathway (Bommareddy et al. 2018). In the study, treatment of MDA-MB 231 human breast cancer cells with 20 and 40 μM concentrations of alpha-santalol inhibited the migratory potential when compared to DMSO-treated control cells. Alpha-santalol treatment also blocked the translocation of β-catenin to nucleus and reduced the expression of phospho β-catenin levels in breast

cancer cells (Bommareddy et al. 2018). A different study that investigated the role of a microemulsion formulation of alpha-santalol showed that transdermal/transpapillary delivery of alpha-santalol significantly reduces the tumor incidence and multiplicity in a rat chemical carcinogenesis model of breast cancer (Dave et al. 2017). Most recently, a clinical study demonstrated the effectiveness of turmeric and SWO-based cream (turmeric extract 16% *w/w*, Sandalwood Oil 0.5% *w/w* in a non-greasy base) against radiation induced dermatitis in women undergoing treatment for breast cancer. Authors from the study attributed the protective effects of curcumin and SWO to their anti-inflammatory and antioxidant ability in modulating cytokines and enhancing wound healing process (Rao et al. 2017).

Prostate Cancer

Comparable to what was previously identified in other cancer models, treatment of prostate cancer cells with alpha-santalol also resulted in induction of apoptosis as verified by DNA fragmentation and nuclear staining of apoptotic cells by DAPI (Bommareddy et al. 2012). The apoptotic and growth inhibitory effects of alpha-santalol were evident in both PC-3 and LNCaP cells, irrespective of their androgen or p53 status, and results were concentration and time-dependent. The alpha-santalol-induced apoptotic cell death and activation of caspase-3 were significantly diminished in the presence of pharmacological inhibitors of caspase-8, 9 (Bommareddy et al. 2012).

The *in vivo* efficacy of alpha-santalol against prostate cancer development was first demonstrated in a xenograft mouse model for prostate cancer (Saraswati et. al. 2013). In the same study, the effects of alpha-santalol against angiogenesis was investigated employing human umbilical vein endothelial cells (HUVECs) and prostate tumor cells (PC-3 or LNCaP) *in vitro*. Alpha-santalol inhibited migration of endothelial cells in a dose-dependent manner, and inhibited the invasion of HUVECs and capillary tube formation. It significantly inhibited neovascularization in rat aortic assay *ex*

vivo and sponge implant angiogenesis assay *in vivo*. Alpha-santalol inhibited angiogenesis by targeting the VEGFR2 regulated AKT/mTOR/P70S6K signaling pathway and as a result, reduced tumor growth. In tumor-bearing mice, alpha-santalol treatment not only prolonged their lifespan, but also exhibited fewer adverse effects, clearly demonstrating the antitumor potential of alpha-santalol in prostate cancer treatment. Moreover, the volume and weight of solid tumors in the prostate xenograft mouse model were greatly reduced following treatment with alpha-santalol (Saraswati et al. 2013). Our recent unpublished observations revealed that alpha-santalol targets PI3K/Akt/survivin pathway to induce cell death and that the cell death is enhanced in the presence of a known inhibitor of the pathway (Bommareddy et al. unpublished observations).

ANTI-INFLAMMATORY PROPERTIES

In close association with the cancer preventive and anti-tumor properties, anti-inflammatory effects of SWO and alpha-santalol are a commonly studied characteristic. One study specifically looked at the ability of alpha-santalol to alter the expression of various cytokines and chemokines, particularly in skin tissue models (Sharma et al. 2014). The study identified that over 20 of the 26 observed inflammatory mediators stimulated through human dermal fibroblast and neo-epidermal keratinocyte exposure to lipopolysaccharides were significantly suppressed when alpha- or beta-santalol was administered with ibuprofen. Additionally, the SWO obtained from East India SandalWood Oil (EISO) showed increased inflammatory suppression, correlated to a higher santalol concentration. Apart from the observed cytokines, both alpha- and beta-santalol suppressed lipopolysaccharide mediated action of the arachidonic acid pathway, while decreasing prostaglandin E2 and thromboxane B2. Overall, the anti-inflammatory characteristics of SWO and alpha-santalol suggest that it may have a place in the use of topical anti-inflammatory products.

A later study performed by the same group sought to observe the effect of SWO on decreasing inflammatory and proliferative characteristics of psoriasis - a disease identified by discomforting, inflammatory plaques from keratinocyte hyperproliferation. The study compared both *in vitro* normal skin tissue and psoriatic skin tissue models, finding that there was no effect experienced on normal skin (Sharma et al. 2017). In the psoriatic skin samples, a marked decrease in the expression of key inflammatory factors was shown in addition to decreased proliferation via histological observation (Sharma et al. 2017). These results again suggest that SWO and particularly alpha-santalol, has observable anti-inflammatory effects.

In a third study performed by this group, observed EISO's effects on PDE regulation of proinflammatory cytokine production. The study demonstrated the effects of EISO on *in vivo* samples of skin tissue affected with atopic dermatitis, as well as *in vitro* human dermal fibroblasts and bronchial epithelial cells. In the sample affected with atopic dermatitis, the EISO treatment showed reduced PDE4 expression, which is a PDE isoform that is overactive in chronic relapsing inflammatory disease (Sharma et al. 2018). Additionally, in the *in vitro* sample, EISO was found to directly inhibit PDE enzymatic activity (Sharma et al. 2018). These results demonstrate EISO's anti-inflammatory effects and its ability to serve as a possible treatment for chronic inflammatory conditions.

In a similar study, five groups of rats were randomized into different dietary groups characterized by the implementation of various natural oils which were compared to SWO - the primary source of alpha-santalol. The study found that 10% SWO in soybean oil as a dietary addition for 8 weeks resulted in higher total n-3 polyunsaturated fatty acids (PUFA's) but a decreased ratio of n-6:n-3 PUFAs in adipose and liver tissues alike (Li et al. 2013). Further, the study reviewed a correlation between n-6:n-3 ratios in rat liver and plasma concentrations and concluded that n-6 PUFAs are closely related to the expression of proinflammatory cytokines such as prostaglandins and leukotrienes. Conversely, n-3 PUFAs were associated with anti-inflammatory processes and reduced expression of these cytokines. This study ultimately concluded that SWO, a source for alpha-santalol, may be associated with anti-inflammatory activity. PUFA's,

particularly n-3 series such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which antagonizes the effects of n-6 PUFAs, have been closely associated with decreasing cardiovascular disease and risk, as well as anti-inflammatory properties (Siriwardhana et al. 2012). A summary of the anti-inflammatory effects is presented in Table 2.

ANTIHYPERGLYCEMIC PROPERTIES

Alpha-santalol has been shown to have antihyperglycemic properties. In one study alpha-santalol, SWO, or glibenclamide, an antihyperglycemic agent, was intraperitoneally (i.p.) administered to mice with chemically induced diabetes. The mice receiving alpha-santalol and SWO showed an increased body weight, decreased water intake, increased liver weight, glycogen, and liver protein. Blood glucose levels were significantly decreased, with all measured variables comparable to those of glibenclamide. The study also showed that mice under oxidative stress due to administration of D-galactose had improved parameters when alpha-santalol and SWO were administered. These results were similar to those of alpha-tocopherol, a strong dietary antioxidant. The study also indicated that some of the beneficial attributes of alpha-santalol were enhanced by other components of SWO (Misra et al. 2013). In a different study, oral administration of sandalwood pet ether to rats with chemically induced diabetes reduced blood glucose by 140 mg/dL over 60 days. The group treated with known antihyperglycemic agent, metformin, showed only a 70 mg/dL decrease. Additionally, total cholesterol, low density lipoprotein (LDL), and triglyceride levels were decreased in the diabetic rats, while high density lipoprotein (HDL) levels increased. These results show the possible antihyperglycemic and antihyperlipidemic properties of alpha-santalol and SWO (Kulkarni et al. 2012). A summary of the anti-hyperglycemic effects is presented in Table 3.

Table 2. Anti-inflammatory effects

Lead Author	Year	Sample	Effects studied	Treatment	Results
Li	2013	Sprague-Dawley rats (male)	Natural oil effects on fatty acid profiles and inflammatory factors	Dietary compound of 10% soybean oil, 10% olive oil, 10% safflower oil, 10% linseed oil or 8% SWO, each blended with 2% soybean oil for 8 weeks	SWO caused a higher total n-3 PUFAs concentration. A correlation was found between n-6:n-3 rations in rat liver and plasma concentrations that n-6 PUFAs is closely related to the expression of proinflammatory cytokines.
Sharma	2014	Human fibroblasts and keratinocytes	Inflammatory mediator stimulation through exposure to lipopolysaccharides	Alpha- or beta-santalol with ibuprofen	Over 20 of the 26 observed inflammatory mediators were significantly suppressed when alpha- or beta-santalol was administered with ibuprofen, while increased inflammatory suppression correlated to a higher santalol concentration.
Sharma	2017	Human psoriatic skin (*in vitro*)	Inflammatory and hyperproliferative effects of psoriasis	EISO	A marked decrease in the expression of inflammatory factors ENA-78, IL-6, IL-8, MCP-1, GM-CSF, and IL-1β was shown in addition to decreased proliferation
Sharma	2018	Human dermal fibroblasts and bronchial epithelial cells (*in vivo*, *in vitro*)	Inflammatory mediation as a phosphodiesterase inhibitor	EISO - (0.001-0.002%)	EISO suppressed PDE activity, PDE4, and 7 transcription levels, NF-κB activation, and pro-inflammatory cytokine/chemokine production.

Table 3. Anti-hyperglycemic effects

Lead Author	Year	Sample	Effects studied	Treatment	Results
Kulkarni	2012	Streptozotocin-induced diabetic rats	Antihyperglycemic and anithyperlipidemic potentials	Twice daily i.p. injection of SWO (10 micro-g/kg) for 60 days	Decreased blood glucose levels and decreased total cholesterol
Misra	2013	Alloxan-induced diabetic Swiss albino male mice	Antihyperglycemic	Daily i.p. injection of alpha-santalol (100 mg/kg) and sandalwood oil (1 g/kg) for 1 and 2 weeks	Improved parameters including increased body weight and decreased blood glucose levels. Oxidative stress parameters also improved. The beneficial effects of alpha-santalol were enhanced when SWO was used.
		D-galactose mediated oxidative stress induced male mice	Antioxidant		

NEUROLOGICAL EFFECTS

Second only to its anti-tumor properties, alpha-santalol's neurological effects are highly researched - many studies have shown that alpha-santalol has sedative properties. One of the first animal studies examining the neurological effects of SWO and its constituents via i.p. injection in mice indicated that alpha- and beta-santalol were responsible for the neurological activity associated with the oil. These constituents increased the levels of homovanillic acid, 3,4-dihydroxyphenylacetic acid and/or 5-hydroxyindoleacetic acid in the brain conveying a sedative effect with similar activity to chlorpromazine, an antipsychotic medication (Okugawa et al. 1995). A study undertaken to investigate axiolytic-like activity of hexane - extracted SWO on mice subjected to water-immersion stress

revealed that inhalation SWO at 4 µl/l air resulted in significant anxiolytic-like activity (Satou et al. 2014). In another study focusing on emotional behavior induced by alpha-santalol, the compound was administered to mice i.p. and through inhalation. Only i.p. administration resulted in a significant decrease of locomotor activity suggesting sedation, but not anxiolytic activity (Satou et al. 2015). A closer examination into the possible mechanisms of the compound was performed using naloxone and alpha-santalol to inhibit writhing in mice caused by acetic acid. It was observed that alpha-santalol caused an analgesic effect via $\boldsymbol{\delta}_2$-opioid receptor indicating that alpha-santalol may be an effective pain treatment without the potential for addiction. Alpha-santalol was also shown to be a dopamine D2 and serotonin 5-HT2A receptor antagonist, suggesting an antipsychotic effect with a similar but weaker activity compared to chlorpromazine (Okugawa et al. 2000). Another animal study observed the effect of alpha-santalol on sleep-deprived rats. Inhalation of the compound caused a significant decrease in time the rats were awake and increased non-rapid eye movement during sleep. There was no significant difference in the effect of alpha-santalol upon obstruction of the olfactory system; suggesting alpha-santalol exhibits its effects through the circulatory system (Ohmori et al. 2007).

A human study examining the sedative effects of alpha santalol was also performed. Thirty-six healthy volunteers between nineteen and thirty-two years old were divided into three groups. Baseline data was first gathered through a placebo-only trial. The groups were administered one ml of SWO, alpha-santalol, or peanut oil transdermally. The results of the study showed a significant decrease in pulse rate and eye blink rate for the SWO and alpha-santalol groups (Hongratanaworakit et al. 2004). Another human study focused on the inhalation of SWO and alpha-santalol. Similar parameters were measured, but elevated pulse rate, skin conductance, and systolic blood pressure were found. Correction analyses revealed that these effects were mainly due to the perceived odor quality of the compounds (Heuberger et al. 2006).

A recently concluded study employing the genetics of *Caenorhabditis elegans* model for the first time demonstrated the potential neuroprotective

mechanisms of EISO and its major active principles, alpha- and beta-santalol (Mohankumar et al. 2018). The study showed that EISO and its components protected *C. elegans* from 6-hydroxy dopamine (6-OHDA)-induced neurodegeneration and other Parkinson's disease associated pathologies without altering the overall physiological functions. In addition, the study also reported antioxidant and neuroprotective effects of EISO, alpha- and beta-santalol capable of reducing the intracellular reactive oxygen species (ROS) levels and neurodegenerative features induced by 6-OHDA. The neuroprotective effects offered by EISO and santalol isomers were dependent on multiple cellular signaling mechanisms linked with longevity and stress resistances including mitochondrial ETC, ERK-MAPK and SKN-1/Nrf2 signaling but not *via* DAF-2/DAF-16 pathway. A summary of the neurological effects is presented in Table 4.

MISCELLANEOUS PROPERTIES

The cardioprotective effects of alpha-santalol are still mainly unknown. One study examined the effects of alpha-santalol when administered transdermally, showing that alpha-santalol may cause a large decrease in systolic blood pressure (Hongratanaworakit et al. 2004). A second study examined the effects of alpha-santalol via inhalation; the study concluded that alpha-santalol did not significantly impact pulse rate or blood pressure, but SWO odor increased pulse rate (Heuberger et al. 2006).

Additionally, the effects of alpha-santalol on the liver have been investigated. One study that examined the impact of SWO on liver function by measuring the activity of glutathione S-transferase (GST) and levels of soluble sulfhydryl; found that feeding of 5 µL of sandalwood oil to mice for 10 and 20 days produced a 1.80 and 1.93 fold increase in GST activity, respectively. Feeding of 15 µL for 10 and 20 days produced a 4.73-fold and 6.10-fold increase GST activity, respectively.

Table 4. Neurological effects

Lead Author	Year	Sample	Effect studied:	Treatment	Results
Okugawa	1995	Mice	CNS activity: Sleep, body temperature, pain, motor activity	i.p. injection	The alpha- and beta- santalol components of sandalwood oil were indicated to have a sedative effect
Okugawa	2000	Mice	Impact on acetic acid-induced writhing and dopamine/serotonin reception	i.p. injection	Alpha-santalol acts via a theta 2-opioid receptor. It is also a dopamine and serotonin antagonist.
Hongratanaworakit	2003	Humans	Mental and emotional effects	Transdermal absorption without inhalation	Alpha-santalol appeared to cause sedative effects while sandalwood oil caused behavioral stimulation.
Heuberger	2006	Humans	Arousal	Inhalation	Alpha-santalol produced greater attentiveness and mood whereas sandalwood oil elicited a greater physiological effect
Ohmori	2007	Rats	Sleep-wake cycle	Inhalation (5 X 10 (-2) ppm) and inhalation with shunted olfactory system	Santalol decreased total wake time and increased NREM in rats while sleeping.
Satou	2015	Mice	Emotional Behavior	Inhalation (2 μl/l) and i.p. injection (0.03 mL/kg) of alpha-santalol	A decrease in motor activity was observed after 24 hours of i.p. injection.
Mohankumar	2018	C. *elegans*	Neurotoxic; 6-OHDA and Proteotoxic; (α-synuclein)	Absorption and Incubation	EISO, Alpha- and Beta- santalol reduced intracellular ROS levels and apoptotic features caused by 6-OHDA. Lowered overall neurodegeneration caused by 6-OHDA.

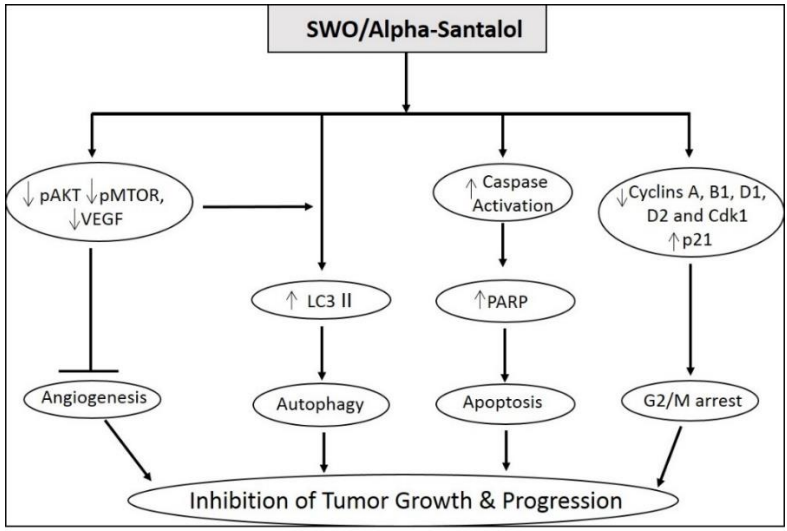

Figure 2. Proposed mechanism of action of SWO and alpha-santalol.

Likewise, feeding of 5 µL for 10 days produced a 1.59-fold increase in sulfhydryl and feeding of 15 µL for 10 days gave a 1.57-fold increase in sulfhydryl (Banerjee et al. 1993).

Some of the antiplasmodial properties of SWO have also been investigated. An *in vitro* investigation indicated the oil is an effective agent against *Plasmodium falciparum*, a known cause of malaria in humans. *In vivo* analysis via subcutaneous administration of SWO to rodents indicated a statistically significant action against *Plasmodium. berghei*, which is known to cause malaria in certain rodents (Fujiaski et al. 2012).

CONCLUSION

The focus of this chapter is to review recent advances in studies identifying the medicinal properties of SWO and alpha-santalol and summarize the *in vitro* and *in vivo* findings, including the results of available and/or completed human studies. Based on the results from pre-clinical studies, it is evident that administration of SWO and alpha-santalol may have protective effects against the development of various diseases.

Studies suggest that SWO and its components are safe and promising therapeutic agents against cancer development with potential to target multiple pathways and modulate expression of markers involved in carcinogenesis. Based on data collected from several studies involving cancer prevention and treatment with SWO and alpha-santalol, the anti-tumor and cancer preventive properties are attributed to their proapoptotic, antiproliferative, antiangiogenic, antioxidant and anti-inflammatory activities. Uniquely, these agents minimize undesirable side-effects and improve patient compliance/quality of life. Studies have also examined the effects of SWO and its components on other health conditions, including diabetes, inflammation, and neurological disorders with promising results. In conclusion, alpha-santalol and SWO have shown promise in many areas of medicinal treatment with properties including anticancer, antidiabetic, antipsychotic, neuroprotective and anti-inflammatory. However, there are certain limitations, such as bioavailability and the concentrations of SWO and alpha-santalol required to exert these beneficial effects. Therefore, studies are warranted to confirm their exact role when used alone or in combination with other agents in disease prevention and treatment.

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