# Lemon Verbena (Aloysia citrodora)

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*Aloysia citrodora* (Verbenaceae), an acknowledged medicinal plant, is traditionally used to treat various diseases, including bronchitis, insomnia, anxiety, digestive, and heart problems.

Keywords: lemon verbena ; plant extracts ; traditional medicine ; antioxidant ; antiproliferation

## 1. Introduction

Worldwide estimation of cancer incidence and mortality, created by the International Agency for Research on Cancer, revealed that 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020. Female breast cancer is the most diagnosed cancer, surpassing lung cancer, with (11.7%) new cases of the total reported cases, followed by lung cancer (11.4%). The global cancer incidences are expected to be 28.4 million cases in 2040, a 47% rise from 2020 [1].

Cancer is a disorder that triggers normal cells' uncontrolled growth and alters the genome (which causes malignant characteristics in normal cells) [2]. This overgrowth progression impairs the normal biological process of healthy cells by the invasion of nearby tissues and metastasizes to distant tissues [3]. The first options of treatment are chemotherapy, radiation therapy, and surgery. Many adjuvant therapies and strategies such as quality of life changes, antioxidant supplements, herbal medicines, and remedies based on natural products are continuously growing as promising strategies to augment conventional therapies [4][5][6]. Today, the most reliable option for cancer treatment is chemotherapy, and drug resistance appears as a significant limitation [7][8]. These limitations push researchers to find other sources for treatment and direct researchers vision into the richest source of treatments from ancient times, to nature.

Another problem is the free radicals that can cause oxidative damage to any sort of cell component. This type of damage in humans can lead to various degenerative disorders, including cancer and cell aging, directing the interest to the antioxidants [9]. Antioxidant molecules, pharmacologically effective and with few or no adverse effects, are currently being explored for preventive care worldwide and in the food industry. Plants are vulnerable to the harmful effects of active oxygen and produce many antioxidant chemicals in addition to tocopherols. Flavonoids, several other phenolic compounds, and polyphenolics are examples of these substances [9].

All societies around the world have consumed medicinal plants. In the United States and Europe, natural products or their derivatives account for nearly (50%) of all prescription drugs [10]. Many drugs used to treat cancer, infections, and other disorders are entirely derived from plants or are synthetic/semi-synthetic derivatives of plants [11]. According to the World Health Organization (WHO), (11%) of drugs are derived entirely from plants, with a significant percentage of synthetic medications derived from natural precursors [12]. Showing the increasing importance of natural products in drug development.

Approximately 150 plant herbs are still used as a source of traditional herbal therapy in Jordan [13]. One of these herbs is Lemon verbena, scientifically known as *Aloysia citrodora* Paláu, an acknowledged medicinal plant [14]. *A. citrodora* has a broad range of medicinal, cosmetic, aromatic, and culinary applications [15]. *A. citrodora* extracts and preparations are mostly reported to have antioxidant and anti-microbial biological activities [16][17][18][19]. Phytochemically, the extracts of *A. citrodora* were found to have an excessive amount of phenolics such as phenylpropanoids, flavonoids, lignans, tannins, and a variety of other nonphenolic compounds [20]. Generally, the major compounds of the oil of *A. citrodora* were the citral isomers geranial and neral [21][22]. However, limonene, 1,8-cineol, β-caryophyllene, citronellal, and citronellol were frequently listed as major ingredients. Furthermore, studies showed that the oil composition is affected by numerous factors like plants' genotype, environmental factors, and growth conditions [23][24].

## 2. Antioxidant Activity

Natural antioxidants found in medicinal herbs are responsible for reducing or inhibiting the harmful effects of oxidative stress and reactive oxygen species (ROS), which have been detected in almost all cancer types and promote their progression, development, survival, and boost its metastatic ability. Novel therapeutic approaches are needed to control the intracellular ROS signaling production and ROS-induced tumor. Then again, the antioxidants could avert initiation events of cancer, where ROS considered a crucial part [25]. The hydroxyl groups of the active compounds permit a scavenging effect on various reactive oxygen and nitrogen species and provide a powerful antioxidant effect [26].

Free radical scavenging activity is determined through several available procedures. However, the radical-scavenging DPPH test has attracted the most attention [27]. It has been utilized for evaluating the antiradical properties of extracts very often because it can accommodate many samples in a short amount of time and is sensitive enough to detect active components at low concentrations [28].

Ethanol extracts were found to be the most potent scavengers against the DPPH radical, followed by water extract. It could be due to the amount of phenols and flavonoids [29]. Depending on the TPC experiment, phenols are abundant in these extracts. Although TPC measures all phenolics, some heterogeneous phenolic compounds could respond differently. F–C values are found to be higher than those obtained by other methods such as HPLC-MS/MS and HPLC-UV [30][31]. A study implied that the antioxidant capability of an extract is determined not only by the amount of polyphenols present but also by the nature of the antioxidants molecules [32]. The potent antioxidant extracts are rich in polyphenols. Numerous investigations have found a link between phenolic composites and radical-scavenging capacity [33][34]. A significant linear correlation was found between the content of phenolic compounds and the extracts' antioxidant activity in a few plants [35].

Ethyl acetate extract showed lower activity against DPPH. The previous findings on the extract components revealed potent activity when assessed solely [36][37][38]. This activity could be affected by many factors. The constituent of the reacting mixture differs in molecular size, polarity, solubility, chemical structure, concentration, and molecular ratio. The interactions between the components (not only with the active compounds) result in additive, synergistic or antagonistic effects [39]. As in the case of ethyl acetate extract, most probably an antagonistic effect between its components causes the drop in the activity.

The free-radical scavenging activity of *A. citrodora* EO was also assessed in this entry. The oil did not exhibit any radical scavenging activity at all concentrations examined. Abuhamdah, R., & Mohammed (2014) conducted a study on Jordanian origin *A. citrodora* fresh and dried leaves identified 83 compounds of essential oil chemical composition. The highest components were limonene (12.14%) caryophyllene oxide (10.44 %), curcumene (9.17 %), spathulenol (7.16 %), 1,8-cineole (7.94%), followed by geranial (4.03 %) and neral (2.55) [40]. Another study of the EOs obtained from dried or fresh Jordanian *A. citrodora* leaves identified limonene, neral, and geranial as major components. They have also identified in low amounts α-pinene, α-terpinene, sabinene, linalool, and caryophyllene [41]. A study of Jordanian *A. citrodora* aerial parts by Hudaib et al. (2013) revealed limonene (17.7%) as the highest constituent, together with the citral isomers (neral: 9.8% and geranial: 10.1%), representing more than one-third of the oil. Other major components identified in *A. citrodora* oil included mainly 1,8-cineole (11.7%), α-curcumene (6.3%), and the oxygenated sesquiterpene components spathulenol (4.6%) and caryophyllene oxide (3.1%) [42].

Studies from several countries in the literature revealed the presence of citral isomers (neral and geranial) in *A. citrodora* essential oil [43][44][45][46][47]. However, the present entry results did not indicate the occurrence of any common major detected compounds (e.g., neral, geranial, thujone, citronellal, carvone). In a recent article of the *A. citrodora* EOs from two separate localities, the antioxidant activity of the plant containing more citral resulted in more potent antioxidant activities [48]. The literature emphasizes that various factors could affect EOs composition, such as the age-related stage of the plant, its physiology, and growth conditions. Also, the constituents of the EO could be altered by the isolation and analysis conditions [41][49][50][51].

Another factor affecting EOs antioxidant activity could be the presence of Alpha-pinene and limonene compounds which have poor activity in the DPPH test system. The antioxidative effect of EOs is frequently not attributable to the primary constituents; lesser molecules and synergistic effects may play a substantial role in the activity [52]. Antioxidant activity can be induced by the presence of heteroatom-containing compounds in EOs. Oxygen-containing moieties, such as phenols or hydroxyl, are more effective antioxidants than nitrogen-containing structures like aniline [53].

Variations in the amounts of each compound identified in these extracts had a strong correlation with the extract activity. Jordanian *A. citrodora* in this analysis revealed high variation in its composition compared to studies from different

countries [52][53], even its EO components identified in Jordan 9 years ago [42]. The current findings could indicate the significant effects of these factors (mentioned above) on the quality and quantity of the extracts.

## 3. Antiproliferative Activity

### 3.1. In Vitro Study

Ethyl acetate extract established the highest anti-proliferation activity, followed by the EO and ethanol extract. In contrast, water extract exhibited the lowest activity. The difference in activity could result from the various flavonoids and phenolic substances' chemical structure types found in the extracts. Like in many other plant species, flavonoids might be responsible for the major bioactivities of *A. citrodora*, such as antimicrobial, neuropsychological, antioxidant, cytoprotective, and anti-cancer effects [54]. Flavonoids can occur naturally either as a compound associated with sugar in conjugated form (glycosides) or without attached sugar as aglycones. The presence or absence of sugar moiety can affect the flavonoids' solubility, reactivity, and stability [55]. Overall, pharmacokinetic properties can have a major impact on the health-promoting effects of phytochemicals [26].

To the best of researchers knowledge, the current entry is the first attempt to assess the antiproliferative property of the aerial parts of *A. citrodora* extracted by ethyl acetate (EA) as solvents that had the ability to extract various non-polar active compounds. The LC-MS analysis revealed naringenin (flavanones) as the most abundant compound in EA extract (25.22%). Naringenin is insoluble in water and soluble in organic solvents, like alcohol [56][57]. It has been found in several plants possessing various biological activities like antioxidant, antitumor, antiviral, antibacterial, anti-inflammatory, and cardioprotective effects [36][58][59][60]. In many reports, Naringenin exhibited antiproliferation effect, ability to inhibit cell growth, increase AMP-activated protein kinase phosphorylation, CyclinD1 expression down-regulation, and cell death induction. Other reports include promising results for prostate cancer, melanoma, and gliomas-brain cancer [61][62][63].

Another major detected component was 5,6,4′-Trihydroxy-7,3′-Dimethoxyflavone (5-TDMF) (23.67%)**.** It is proven to have potent antioxidant activity in vitro and ex vivo. One study demonstrates that 5-TDMF has potent antioxidant and anti-inflammatory effects without cytotoxicity observed. The same study suggested that (5-TDMF) could block LPS-induced NF-κB translocation and iNOS and COX-2 expressions by inhibiting the mitogen-activated protein (MAP) kinase and MAPK/ERK signaling pathways. Suggesting potential novel chemo-preventive anti-inflammatory agents [38]. Still, a lack of detailed studies is noticed regarding its molecular mechanism that controls its activities, such as binding to a particular protein structure [64].

Hispidulin (HIS) (22.61%) is another detected compound considered a monomethoxy and trihydroxy flavone compound. In vitro investigations suggested the capability to affect the activation of JNK, p38, and NF-κB [65]. Also, it could control helenalin-induced cytotoxicity [66]. HIS has been reported in numerous studies to have potential antimutagenic, antioxidative, and anti-inflammatory effects [67][68][69]. Besides, many reported anticancer activity against multiple cancer cell lines such as gastric, pancreatic, ovarian, gallbladder, and colorectal. In addition to glioblastoma renal carcinoma, acute myeloid leukemia, Glioblastoma Multiforme, hepatocellular carcinoma cancers [69][70][71][72][73][74][75][76][77][78][79][80][81][82]. In colon cancer, HIS was noticed to inhibit the hypoxia-generated epithelial-mesenchymal transition, which had significantly enhanced the cytotoxicity of anticancer drugs against cancer cells [70].

Other detected components include Eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone) (13.24%). That is known to possess promising antiproliferative, anti-inflammatory, modest antioxidant, neuroprotective, anti-allergic, and cardioprotective activities [37][83][84][85][86][87][88][89][90][91]. Several studies on eupatilin have explained its anti-cancer property due to its promising capacity to prompt apoptosis in different cancer cell lines [83][84][92][93][94][95][96][97][98]. Same as baicalein bioflavonoid (S7.84%), which can arrest cancerous cells at the G2/M and G1/S cell cycle phases [56]. It also decreases cell proliferation or induces apoptosis in multiple myeloma and cancer types. It has been demonstrated to inhibit cancer cell migration and invasion in many studies [99][100][101][102][103][104].

No available studies have been found related to 5,7-Dihydroxy-2′-Methoxyflavone (2′-Methoxychrysin) (4.91%) activity, although this compound has been separated previously from many species [105][106]. This compound has the physicochemical and pharmacological effects of flavonoids—flavones, and flavon-3-ols [107].

Previous reports revealed different types of flavonoids identified in *A. citrodora* as Skaltsa used column chromatography to isolate several flavonoids from the leaf extract of *A. citrodora* in 1988. Flavone structures were found in all of the purified substances [108]. Subsequently, glycosides of previously isolated flavones were also detected, such as apigenin-7-diglucuronide and chrysoeriol7-diglucuronide in the aqueous extract of *A. citrodora* aerial parts [109]. New flavonoids in the

aerial parts (jaceosidin, nepetin, and nepitrin) have also been reported in recent studies, all of which have flavone structures [110].

The occurrence of these compounds simultaneously could indicate a synergistic, additive, or antagonistic effect on the antiproliferative activity of the extract. Ethyl acetate extract had the most potent antiproliferative effect on all used cancer cell lines ($IC_{50}$ ranging from 136 to 203 µg/mL), even on the normal cell line, which in comparison with cancerous cells and depending on selectivity index, revealed selective toxicity (targeting malignant cells without harmful effect on normal cells) using suitable concentration.

The antiproliferative experiments of the EO of the aerial parts of *A. citrodora* revealed weak activity compared with other studies ($IC_{50}$ ranging from 402 to 633 µg/mL). Oukerrou et al. (2017) demonstrated that *A. citrodora* EO exerted a dose-dependent cytotoxic effect on P815, MCF7, and Vero tumor cell lines, with $IC_{50}$s ranging from 6.60 to 79.63 µg/mL [111]. Another study observed the potent cytotoxic effect of *A. citrodora* EO from two different regions of Palestine on HeLa cell lines. The $IC_{50}$ values were 84.50 and 33.31 µg/mL and compared with Doxorubicin ($IC_{50}$ value of 22.01 µg/mL) [48]. No notable activity was observed regarding ethanol, and aqueous extracts in this entry suggested the presence of weakly active polar compounds or antagonistic interaction between the phenolics and flavonoids components.

### 3.2. In Vivo Study

The ethyl acetate extract of *A. citrodora* was used in the current entry to treat mice implanted with breast cancer cells. A significant reduction in tumor size and a high cure percentage were observed. *A. citrodora* ethyl acetate extract dosage of 0.162 g/kg showed a high reduction in tumor size by 57.97% and many undetected tumors or curing effects of 44.44%. Reduction in the tumor size may be explained by the cytotoxic phytochemicals that exhibited notable effects on tumor cells.

Many studies showed the effect of phytochemical compounds detected in *A. citrodora* with cytoprotective, antioxidant, and anti-proliferation activities [54]. Naringenin, 5,6,4′-Trihydroxy-7,3′-Dimethoxyflavone (5- TDMF), and hispidulin were the most concentrated compounds in ethyl acetate extract. These compounds possess promising antiproliferative activities. In vivo studies with naringenin revealed anti-proliferation activity through a delay of tumor growth on ovariectomized C57BL/6 mice injected with E0771 mammary tumor cells [59]. In another in vivo study using breast cancer cells, observations suggested decreased secretion of TGF-β1 and accumulation of intracellular TGF-β1, and inhibition of TGF-β1 transport from the trans-Golgi network and PKC activity [112]. Also, 5,6,4′-Trihydroxy-7,3′-Dimethoxyflavone (5- TDMF), hispidulin in-vivo anti-proliferation activity, and antioxidant properties were reported [38][113]. These compounds may work synergistically to inhibit cancer either by a direct effect on cancer cells or by an indirect effect through antioxidant activity and other mechanisms [114].

Liver and kidney function enzymes such as ALT, AST, and creatinine are the most reflective parameters of toxicity and safety profile at the therapeutic doses because they are significant in eliminating the drugs through metabolism and excretion [115].

The ALT and AST for the treated group were within the normal range and lower than the normal group, which indicates an acceptable safety profile for all the treatments. These results could be justified because the doses used in this entry chose a dose according to $LD_{50}$ estimation and used Karber method calculation with no toxic outcomes. On the other hand, creatinine levels observed in this entry were higher for the treatment group than the control and normal mice creatinine levels. High kidney enzymes are acceptable as long as they are within the range of normal mice enzymes level. Mainly after no deaths were observed after ten days of treatment, the results might indicate the safety of using the ethyl acetate extract.

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