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Elemental composition, Antimicrobial and Cytotoxic efficacy of *Olea dioica* Roxb. (Oleaceae)

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Abstract:

The present study was conducted to determine of elemental composition, antimicrobial and cytotoxic efficacy of leaves of *Olea dioica* Roxb. (Oleaceae). The powdered leaf material was digested in ultrapure nitric acid in microwave digester and the elements in digested sample were estimated using Inductively Coupled Plasma with Optical Emission Spectroscope (ICP-OES). The leaf powder was extracted using methanol in Soxhlet assembly and the extract was subjected to phytochemical screening. Antimicrobial activity was assessed against six bacteria and two fungi by Agar well diffusion method. Cytotoxic activity was tested against two cell lines *viz.*, HT29 (human colon carcinoma) and MDA-MB-231 (Human breast cancer) by MTT assay. Of the elements estimated, the content of calcium and manganese were high among macro and microelements respectively. The extract caused dose dependent inhibition of bacteria and fungi tested. Gram positive bacteria were inhibited to high extent than Gram negative bacteria. Among fungi, *Candida albicans* was more susceptible to the extract. The extract inhibited the growth of HT-29 and MDA-MB-231 cells in a concentration dependent manner with IC_{50} value of 131.87 and 140.25µg/ml respectively. The leaves of *O. dioica* can be a good source of elements needed for normal physiology of the body. The leaf showed marked antimicrobial and cytotoxic potential and thus, it could be used to treat infectious diseases and cancer. Isolation and bioactivity of active principles present in the leaf are under study.

Keywords: *Olea dioica*, Elements, ICP-OES, Agar well diffusion, Cytotoxicity, MTT

Introduction

Human beings require a number of organic and inorganic compounds in order to meet the requirements for daily activities. Macronutrients such as carbohydrates, fats and proteins form the major portion of the diet and are consumed in large amount. Minerals and vitamins form comparatively smaller part and are consumed in much smaller amounts. Mineral elements can be therapeutic, or can contribute to the normal health. Some 25 elements have been identified as essential for keeping human health; therefore, the study of elements in food and medicinal plants is of great interest. Plant materials form a major portion of diet and their nutritive value is important. Mineral elements play an important role in the normal physiology of the body and their absence or insufficiency leads to adverse effects on the body. Minerals serve as components of enzymes, regulate cellular energy transduction,

gas transport, antioxidant defense, membrane receptor functions, second-messenger systems and integration of physiological functions. Hence mineral elements are involved in the regulation of use of macronutrients[1-3].

The discovery of antibiotics is considered rightly as one of the most significant health-related events of modern times, not only for their impact on the treatment of infectious diseases but also for other biological activities such as anticancer, growth promotory etc. Antibiotics have revolutionized the field of medicine in many respects and their discovery and subsequent use saved countless lives. However, the successful use of any agent is compromised by the potential development of tolerance or resistance to the agent. Regrettably, this situation is encountered more often in case of antibiotics as the overuse and abuse of antibiotics led to the development of resistance among microorganisms. Microorganisms such as *Staphylococcus aureus, Pseudomonas aeruginosa, Mycobacterium tuberculosis* and others have developed resistance against a wide range of antibiotics. Hence, there is a constant need for the development of new antimicrobials from natural sources, particularly from plants. Plants produce a variety of substances and most are secondary metabolites which serve in defense against predators, responsible for typical odors, and characteristic pigmented nature of plants. Many of these are extensively used as medicinal compounds for treatment of various ailments in different parts of the world especially in under-developing and developing countries[4-6].

Cancer is serious clinical problem and poses a significant social and economic impact on the health care system. It is the second largest cause of death in the world. It affects more than 200 cell types and major characteristic is the lack of control of the cell proliferation, differentiation and death, invading organs and tissues. Even after advancements in diagnosis, prevention and therapy, cancer still affects millions of patients worldwide, reduces their quality of life and is still one of the leading causes of death in the world[7-9]. Chemotherapy is the principal mode of treatment for various cancers. However, development of resistance to chemotherapeutic drugs hampers effective killing of the cancer cells, resulting in tumour recurrence. Also, patients suffer from serious side-effects such as cardiac and other toxicities[10]. Hence, a major portion of current pharmacological research is concentrated on the design of drugs against cancer. Plants produce a variety of structurally diverse bioactive compounds and hence plant kingdom is a potential source of phytoconstituents with antitumor and cytotoxic activities. Many compounds possessing marked anticancer activity such as vincristine, vinblastine, taxol, campothecin and others have been isolated from plants and have dramatically improved the effectiveness of therapy against some dreadful cancers[9].

Olea dioica Roxb. (Oleaceae) is a medium sized evergreen tree and is common in evergreen and semi-evergreen forests of South India particularly throughout Western Ghats. Bark is brownish in color and rough. Leaves are simple, opposite, elliptic lanceolate with serrate margin, leaf apex is acute, inflorescence has axillary divaricate panicles, flowers are polygamodioecious and creamy white in color, fruit is fleshy drupe, ellipsoidal with one seed, when ripen black in color. Flowering occurs in February till April[11]. In a previous study, potent antioxidant efficacy of extracts of leaves was reported[12]. In South west Maharashtra, the ash of fruit is mixed with roots of

Hemidesmus indicus and used for skin diseases. Bark and fruit paste is applied in rheumatism; decoction is used to wash old wounds and given in fever[13]. Ripe fruits are traditionally used by the tribes in the Parambikulum wildlife sanctuary, Kerala, India[14]. A detailed literature survey revealed that the elemental composition, antimicrobial and cytotoxic activity of *O. dioica* is not yet carried out. Hence, in this study, we have determined the elemental composition, antimicrobial and cytotoxic activity of leaves of *O.dioica*.

MATERIALS AND METHODS

Chemicals

Nitric acid (ultrapure, metal free), Dimethyl sulfoxide (DMSO) and multi-elemental standard solution containing all elements estimated in the study was purchased from Merck, Germany. Nutrient agar, Nutrient broth, Sabouraud dextrose agar, Sabouraud dextrose broth, Dulbecco's Modified Eagle Medium (DMEM), Penicillin, Streptomycin, fetal bovine serum and methanol were purchased from HiMedia laboratories, Mumbai.

Collection of plant material

The plant was collected in the month of May 2012 from a place called Kanivebagilu, Hosanagara (Taluk), Shivamogga (district), Karnataka and authenticated by Prof. K.G. Bhat, Udupi, Karnataka, India. The voucher specimen (SRNMN/MB/PPV-03) was deposited in the department herbaria for future reference. The leaves were washed well in order to remove extraneous matter, shade dried, powdered mechanically.

Determination of elemental composition

A known quantity of the powdered leaf material (1gm) was digested in 10ml of ultrapure metal free nitric acid in a microwave digester (CEM). After complete digestion, the content was diluted to 25ml with distilled water. Elemental analysis was performed using Inductively Coupled Plasma with Optical Emission Spectroscope (ICP-OES). The digested sample was aspirated in ICP-OES (Agilent Technologies 700series, United States) to estimate macroelements *viz.*, calcium (Ca), potassium (K) and magnesium (Mg) and microelements *viz.*, manganese (Mn), iron (Fe), zinc (Zn), lithium (Li) and copper (Cu) present in leaf. The calibration standards were prepared by diluting the stock multi-elemental standard solution (1000 mg/L) in nitric acid. Instrument configuration and general experimental conditions are summarized in Table 1.

Table 1: ICP-OES Operation conditions

Parameter	Value
Power (kW)	1.2
Plasma flow (L/min)	15.0
Auxiliary flow (L/min)	1.50
Nebulizer flow (L/min)	0.75
Sample flow rate (L/min)	1.5
Replicate read time (s)	3.00
Instrument stabilization delay (s)	15.0
Sample uptake delay (s)	10.0
Pump rate (rpm)	15.0
Rinse time (s)	10.0
Spray chamber	Cyclonic type
Elements, wavelengths (nm)	Ca (422.673), Cu (327.395), Fe (238.204),K (766.491), Mg(279.553), Mn (257.610), Zn (213.857), Li (670.783)

Extraction and phytochemical analysis

A known quantity of powdered leaf material (100gm) was subjected to soxhlet extraction and extracted exhaustively with methanol for 48 hours. The extract was filtered through 4-fold muslin cloth followed by Whatman No. 1 and concentrated in

vacuum under reduced pressure and dried in the desiccator[15]. The extract was screened for the presence of alkaloids, flavonoids, steroids, glycosides, triterpenoids, saponins and tannins by standard phytochemical tests[16,17].

Antimicrobial activity of extract

Antibacterial activity was assessed against four Gram negative bacteria *Escherichia coli*, *Shigella flexneri*, *Ralstonia solanacearum* and *Xanthomonas campestris* and two Gram positive bacteria *Bacillus cereus* and *Staphylococcus aureus*. Antifungal activity was tested against *Candida albicans* and *Cryptococcus neoformans*. The test bacteria were aseptically inoculated into sterile nutrient broth tubes and incubated for 24 hours at 37°C. The test fungi were inoculated into sterile Sabouraud dextrose broth tubes and incubated for 48 hours at 37°C. The extract was dissolved in 10% DMSO to get desired concentrations *viz.*, 10, 25 and 50mg/ml. Streptomycin was used as standard antibiotic (1mg/ml of sterile distilled water). Agar well diffusion method was performed to screen antimicrobial efficacy of extract[15]. The broth cultures of test bacteria and test fungi were aseptically swabbed on the sterile Nutrient agar and Sabouraud dextrose agar plates uniformly. Using a sterile cork borer, wells of 0.6 cm diameter were punched in the inoculated plates and 100 µl of different concentrations of extract, standard (Streptomycin, 1mg/ml of sterile distilled water) and DMSO (10%) were filled into the respectively labeled wells. The plates were kept in room temperature for an hour and then incubated at 37°C for 24 hours (for bacteria) and 48 hours (for fungi). The presence of zones of inhibition around the wells was observed and interpreted as an indication of antimicrobial activity.

Cytotoxic activity of extract

Cell lines *viz.*, HT-29 (human colon carcinoma) and MDA-MB-231 (Human breast cancer) were used to screen cytotoxic potential of extract. The HT-29 and MDA-MB-231 cells were maintained in DMEM, supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL of penicillin and 100µg/mL streptomycin. The cells were maintained at 37°C in a humidified atmosphere under 5% CO_2. The extract was dissolved in DMSO at 20 mg/ml as stock solution which was then diluted with DMEM to desired concentrations ranging from 10 to 200µg/ml. The final concentration of DMSO in each sample did not exceed 0.1% v/v in both control and test. Cell viability was assessed by MTT (3'-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide) assay, which is based on the reduction of MTT into formazan dye by active mitochondria[18]. In this assay, the cells were placed in 96-well plates at a density of 5×10^4 cells/well in culture medium that contained 10% FBS and then incubated at 37°C under 5% CO_2. After 24 hours, the cells were washed and placed in culture medium with different concentrations of extract for 48 hours. Then, 20µL of MTT solution (5mg MTT/mL in phosphate buffered saline) was added to each well of a microtitre plate and then incubated for 4 hours at 37°C. After washing, the formazan dye precipitates, which are proportional to the number of live cells, were dissolved in 100µL of DMSO. The absorbance (A) at 570 nm was then read using a microtitre plate reader. 5-Fluorouracil was used as positive control. The effects of each concentration were analyzed in triplicate. The rate of cell growth inhibition (CGI) was calculated using the following formula: CGI (%) = ($A_{control}$ –A_{test} / $A_{control}$) X 100%.

Statistical Analysis

All data were expressed as mean±Standard deviation (SD) of the number of experiments (n=3). Past software version 1.92 was used. The IC_{50} values were calculated by Origin 6.0 software.

Results

Elements estimated in leaf material

The elemental composition of the powdered leaf material was determined using ICP-OES. Among macroelements, calcium was detected in high amount (5638.62±0.05 ppm) followed by potassium and magnesium. In the case of microelements, the concentration of manganese (194.56±0.01 ppm) was highest followed by iron, lithium, copper and zinc (Table 2).

Table 2: Elemental content of *O. dioica* leaves

Element	Content (ppm)
Calcium	5638.62±0.05
Potassium	3820.48±0.05
Magnesium	1615.25±0.09
Manganese	194.56±0.01
Iron	148.52±0.01
Lithium	24.26±0.03
Copper	16.50±0.05
Zinc	13.80±0.05

Phytochemical constituents detected in extract

Preliminary phytochemical analysis of the extract showed the presence of alkaloids, steroids, saponins, flavonoids and tannins. Glycosides and triterpenoids were not detected.

Antibacterial activity of extract and standard

The extract showed dose dependent inhibition of test bacteria as revealed by the presence of zone of inhibition around the well (Table 3). Highest inhibition of was observed in case of Gram positive bacteria than Gram negative bacteria. Inhibition caused by Streptomycin was higher than that of leaf extract. *X.campestris* and *S.aureus* were inhibited to high extent among Gram negative and Gram positive bacteria respectively. *R.solanacearum* was not affected by the lowest concentration of the extract tested. DMSO did not cause inhibition of bacteria. Overall, the susceptibility to extract and standard was higher in case of Gram positive bacteria.

Table 3: Antibacterial activity of extract and standard

Test bacteria	Zone of inhibition in cm (Mean±SD)			
	Extract			Streptomycin
	10mg/ml	25mg/ml	50mg/ml	
E. coli	0.8±0.03	1.1±0.50	1.5±0.03	3.3±0.03
S. flexneri	0.8±0.50	1.2±0.50	1.7±0.30	3.5±0.01
X. campestris	1.3±0.50	1.7±0.05	1.9±0.85	3.5±0.09
R. solanacearum	0.0±0.0	0.8±0.10	1.4±0.30	2.8±0.10
B. cereus	1.4±0.09	1.9±0.10	2.3±0.50	3.9±0.15
S. aureus	1.5±0.05	1.9±0.05	2.5±0.10	4.5±0.20

Antifungal activity of extract and standard

The efficacy of methanol extract to inhibit *C.albicans* and *C.neoformans* was shown to be dose dependent and the result is represented in the Table 4. Inhibition caused by standard (Fluconazole) was marked when compared to extract. Overall, the susceptibility to methanol extract and standard was higher in case of *C.albicans* when compared to *C.neoformans*. There was no inhibition of test fungi in case of control (DMSO).

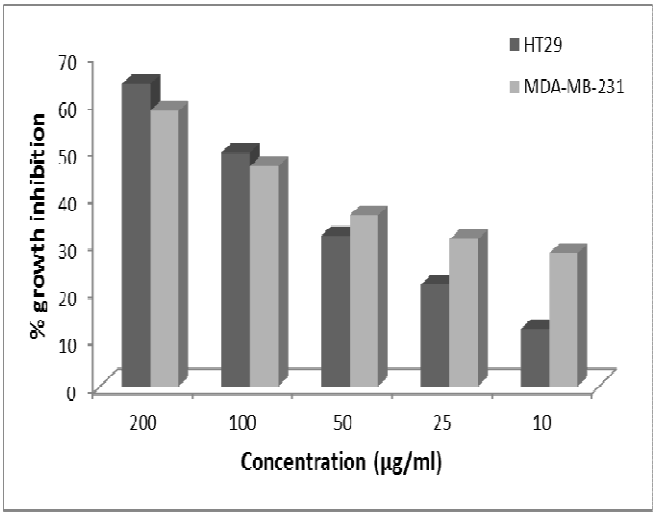
Table 4: Antifungal activity of extract and standard

Test fungi	Zone of inhibition in cm (Mean±SD)			
	Extract			Standard
	10mg/ml	25mg/ml	50mg/ml	
C.albicans	0.9±0.03	1.2±0.50	1.8±0.09	4.0±0.20
C.neoformans	0.8±0.50	1.1±0.10	1.6±0.20	3.9±0.10

Cytotoxicity of extract

The survival of HT-29 and MDA-MB-231 cells in the presence of extract and positive control was assessed by MTT method. MTT assay is a simple and reliable technique which measures cell viability and can be used for screening of anti-proliferative agents. Succinate dehydrogenase, a mitochondrial enzyme, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells[19]. Figure 1presents the plot of cytotoxicity (%) versus concentrations of extract (µg/ml). The results showed that the extract inhibited the growth of HT-29 and MDA-MB-231 cells in a dose dependent manner with IC50 value of 131.87 and 140.25µg/ml respectively. However, the growth inhibition caused by extract was lesser when compared to positive control 5-Fluorouracil (IC50 value of 15.94 and 58.18 µg/ml for HT29 and MDA-MB-231 respectively). In the experiment, DMSO was used as extract solubilizing agent at the concentration of 0.1% and it showed no effect on cell proliferation.

Figure 1: Cytotoxic activity of extract against HT29 and MDA-MB-231 cells



Discussion

The sample digestion, with various mixtures of concentrated acids, is required for most of the analytical determinations to estimate metals in plants. Sample digestion is done using different digestion equipment such as open beakers heated on hot plates, block digesters and digestion units placed in microwave ovens. The analytical techniques are mainly based on atomic spectrometry with mono-elemental detection, such as flame atomic absorption spectrometry, graphite furnace atomic absorption spectrometry. Inductively Coupled Plasma with Optical Emission Spectroscope (ICP-OES) has the advantages of high samples throughput due to the multi-elemental determination. Due to its advantages, ICP-OES has become one of the most used techniques for elemental determination, many studies being conducted to validate this method for metals analysis in a large variety of sample types including plant samples[20,21].

The present study focused on the estimation of three macroelements and five microelements in the powdered leaf of *O. dioica* by ICP-OES technique. The content of calcium was high among macroelements. Calcium forms a large portion of the bone, blood and extracellular fluid. It is required for functioning of cardiac muscles, blood coagulation and regulation of cell permeability. It is also important in nerve impulse transmission and neuromuscular system mechanism[2]. Next to calcium, potassium was detected in high quantity. Potassium takes part in ion balance of the body and maintains tissue excitability. It is important as diuretic[2]. The content of Magnesium was least among macroelements in the powdered material.

Magnesium is involved in more than 300 enzyme reactions involved in glycolysis, fat and protein metabolism, ATP hydrolysis and the second-messenger system. It is also a regulator of membrane stability and neuromuscular, cardiovascular, immune and hormonal functions. Its deficiency can lead to muscle weakness, nausea, irritability of muscles and convulsions[1].

The content of manganese was high among various microelements estimated in this study. Manganese is essential for hemoglobin formation but excess is harmful[2]. Next to manganese, the content of iron was found to be high followed by lithium, copper and zinc. Iron is an important trace element serving as a functional component of iron containing proteins such as hemoglobin, myoglobin, cytochromes and enzymes. It is required for delivery of oxygen to the tissues and the use of oxygen at cellular and subcellular levels. Its deficiency leads to anemia, cognitive impairment and immune abnormalities[1]. Lithium has been considered as an essential microelement. Lithium carbonate was used to treat gout and to dissolve urate bladder stones. Lithium carbonate was found to be beneficial in manic depressive illness. Today, lithium carbonate is one of the most widely prescribed psychiatric drugs[22]. Copper is a component of many enzymes. It facilitates iron absorption and incorporation of iron into hemoglobin. Its deficiency can lead to anemia which explains its role in the absorption of iron[1,2]. Zinc is a membrane stabilizer and stimulator of immune system. It is required for the structure and activity of many enzymes and exerts a regulatory role in the body. It is required for nucleic acid and protein synthesis, cellular differentiation and replication, glucose use and insulin secretion. Its deficiency leads to impaired growth and malnutrition, immune abnormalities[1,2].

The infectious diseases caused by bacteria, fungi, viruses and parasites in an individual are due to a complex interaction between the host, the pathogen and the environment. The mortality and morbidity rates have been dramatically decreased after discovery and use of antibiotics. However, indiscriminate use of antibiotics and the ability of microbes to transmit and acquire drug resistance genes led to the development of resistant microbes such as Methicillin resistant *S.aureus*, Vancomycin resistant *S.aureus*, Vancomycin resistant *Enterococci*, Multidrug resistant TB, Fluconazole resistant *C.albicans* and others. There are considerable reports on the progress of drug resistance and is an alarming situation in developed as well as developing countries. The resistance development in microbes has even complicated the treatment of infectious diseases in immunocompromised and cancer patients. In the scenario of emergence of multidrug resistant microbes, it has necessitated the search for new antimicrobials from other sources, particularly from plants. Plants have been considered as a richer source of natural products that have profound effects on human body. The secondary metabolites such as alkaloids, flavonoids, terpenoids, saponins and others, being produced by plants, have shown to be effective in the treatment of infectious diseases. Phytomedicines have no apparent side effects that are associated with synthetic antimicrobials and hence are safer. These metabolites do exhibit activity against target sites which are not used by antibiotics and thus phytomedicines will be active against drug resistant microbes[23-26].

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In our study, the extract of *O. dioica* was effective against test bacteria viz., *E.coli*, *S.aureus*, *S.flexneri* and *B.cereus* which are known to cause food borne infections and others. The extract might be useful in the treatment human diseases caused by these organisms. The extract was also active against two plant pathogenic bacteria *X.campestris* and *R. solanacearum* which have been isolated from infected plants. Thus, the extract can also be used against phytopathogenic bacteria. The extract was effective against *C.albicans* and *C.neoformans* which are known to cause Candidiasis and Cryptococcosis respectively. The curative properties of plants products are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols etc. The preliminary phytochemical tests for extract may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development[15]. Antimicrobial activities of tannins, flavonoids, saponins, terpenoids, alkaloids, steroids and glycosides have been well documented[27-33]. In our study, the methanol extract of *O. dioica* revealed the presence of phytoconstituents *viz.*, alkaloids, steroids, saponins, flavonoids and tannins. The antibacterial activity of extract could be related to the presence of these secondary metabolites.

Over past few years, cancer has been remained a major cause of death and the number of individuals affected with cancer is increasing. Many difficulties have been encountered in cancer therapy and the most frequently are the drug resistance, toxicity and low specificity. Plant derived compounds have played important role in the development of anticancer agents. The anticancer activity of these compounds are related to the regulation of cancer related gene expression, induction of apoptosis, cell cycle arrest and /or DNA fragmentation and inhibition of different cellular enzymes[8,10,34]. A large number of phytochemicals have shown to possess anticancer property and thus are an important source of newer cancer therapy agents. The use of complementary and alternative medicine such as herbal extracts is becoming increasingly popular in the treatment of cancer[34,35]. Antiproliferative activity of crude extracts[7,36,37] and well as phytochemicals namely alkaloids[38], tannins[39], triterpenoids[40], steroids[41], glycosides[42], saponins[43] and flavonoids[44] have been investigated. In our study, the methanol revealed a dose dependent cytotoxicity to two cell lines HT29 and MDA-MB-231. The presence of phytoconstituents viz., alkaloids, steroids, saponins, flavonoids and tannins in the methanol extract of *O. dioica* might be responsible for the observed cytotoxic activity. Hence, there is a great potential for the development of anticancer agents from essentially untapped reservoir of the plant kingdom.

Conclusions

In conclusion, the study showed that the leaf of *O. dioica* contains an appreciable quantity of various elements which produce a significant effect on the body and hence, it can be used as a source of elements. The antimicrobial and cytotoxic activity of the leaf extract could be related to the presence of various secondary metabolites in it and hence the plant may find its possible utilization in the treatment of microbial infections and cancer. Further studies on isolation

of active principles from the extract and their bioactivities are under progress.

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