CHAPTER: 2

REVIEW OF
LITERATURE
RESEARCH WORK CARRIED OUT IN CITRUS MEDICA L.

Around the world researchers worked on different varieties of *Citrus medica* L. The findings related to the present work are summarized in this chapter.

Menichini *et al.* (2011) studied for the first time the chemical composition and the antioxidant, anti-inflammatory and hypoglycemic potential of flowers, leaves, and fruits of *Citrus medica* L. cv Diamante at two maturity stages. Flowers and leaves were characterized by the highest total phenol and flavonoid content. A declining trend was observed during maturity of fruits for both phenols and flavonoids. The antioxidant activity evaluated by the β-carotene bleaching test, showed a strong activity for flowers and endocarp of mature fruits with IC$_{50}$ values of 2.8 µg/ml and 3.5 µg/ml respectively, after 30 minutes of incubation. The mature fruit endocarp (IC$_{50}$ value of 426.0 µg/ml) could inhibit α-amylase with an IC$_{50}$ value two-fold higher than immature fruits. None of the tested extracts affected the proliferation of human skin fibroblasts 142 BR. The obtained results suggest a potential use of *C. medica* L. cv Diamante as new valuable Citrus species with functional properties for food or nutraceutical products.

Yi *et al.* (2010) investigated bioassay-guided anti-inflammatory principles from the stem and root barks of *C. medica* L. var. *sarcodactylis* Swingle has led to the isolation of a new coumarin, namely citrumedin-B and thirty known compounds. The anti-inflammatory components were xanthyletin, nordenatatin, atalantoflavon and lonchocarpol A, which displayed potent nitric oxide (NO)-reducing activity in microglial cells. The structure of this new compound was well elucidated using a combination of 2D NMR spectroscopic technique. These results can be inferred from the treatment of allergic response and inflammatory properties of *C. medica* L. var. *sarcodactylis* Swingle in traditional Chinese medicine.

Menichini *et al.* (2010) evaluated photo-induced cytotoxic activity of *C. bergamia* and *C. medica* L. cv. *Diamante* peel essential oils and two coumarins, bergapten and citropten. Essential oil was obtained by hydrodistillation and
analyzed by GC and GC/MS. To evaluate the cytotoxic activity two melanoma models, amelanotic melanoma C32 and malignant melanoma A375, were used. The most abundant compounds identified in *C. medica* L. cv. Diamante were limonene, γ-terpinene, citral, geranial, β-pinene and α-pinene. A coumarin (citropten) was also identified in *C. medica* L. cv. Diamante. Essential oil of *C. medica* L. cv. Diamante exhibited a selective interesting activity against the A375 cell line with IC$_{50}$ value 89.1 µg/ml, after 100 min exposure to UV irradiation. Antiproliferative activity was not found with citropten. This study suggested that UV irradiation is effective in activating essential oils. This photo-toxicity may be considered as a treatment option in some cases of lentigo maligna or lentigo maligna melanoma.

**Peng et al. (2009)** studied the fruits of fingered citron (*Citrus medica* L. var. *sarcodactylis*) and the major compounds in its essential oil were *d*-limonene (51.24%), γ-terpenene (33.7%), α-pinene (3.40%) and β-pinene (2.88%). The authors performed *in vivo* safety, hypoglycemic and antidiabetic tests in Sprague-Dawley-SPF rats and Wistar DIO rats, respectively. By kinetic analysis on the hypoglycemic patterns of the intraperitoneal glucose tolerance (IPGTT) and the insulin-glucose tolerance tests (IGTT), its insulin secretagogue effect was confirmed. In conclusion, the fruits that concomitantly possess insulin secretagogue and slimming effects would be very beneficial to type 2 diabetes mellitus patients.

**Sood et al. (2009)** evaluated the antioxidative, anti-inflammatory and analgesic potential of *C. medica* peel extract. Antioxidant activity in different solvent systems was evaluated. Ethyl acetate extract of *C. medica* peel (EtCM) showed maximum 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and hydrogen peroxide radical scavenging activity in a dose dependent manner as compared to ascorbic acid. Further, anti-inflammatory and analgesic activities of EtCM (200, 300 and 400 mg/kg) were studied on carrageenan induced inflammatory pain in rats. Anti-inflammatory activity was assessed by measuring paw volume in rats. Analgesic activity was evaluated for its central and peripheral pharmacological actions by
using hot plate, plantar, pin prick and mechanical allodynia tests in rats. EtCM (400 mg/kg) produced significant decrease in paw volume and pain as compared to diclofenac. Essien et al. (2008) reported physicochemical properties and in vitro antifungal effect of the essential oil of the leaves of *C. medica* L. on storage fungi of *Arachis hypogea* L. (groundnut) stored for 6 months using disc diffusion agar method. The oil exhibited a wide spectrum of fungitoxicity, inhibiting all 14 fungus species tested. They suggested that oil could be used as a fumigant against storage fungi for the preservation of stored legume seeds due to its wide range of activity, non-phytotoxicity and long-term persistence of fungitoxicity.

Chauhan et al. (2008) evaluated efficacy of *Citrus medica* Linn. against ethylene glycol induced urolithiasis in Wistar rats. In this study *C. medica* showed significant activity against urolithiasis which may be because of its diuretic activity, ability to increase inhibitors (citrate and magnesium) level and decrease promoters (calcium, oxalate, inorganic phosphate, uric acid) levels.

Jayaprakasha & Patil (2007) in their study isolated antioxidant fractions from two citrus species such as Citron (*C. medica*) and blood orange (*C. sinensis*). Antioxidant fractions were extracted from mature, ripe fruits using five different solvents in a Soxhlet extractor. The total phenolic content of the extracts was determined by Folin-Ciocalteu method. MeOH : water (80:20) extract of citron and acetone extract of blood orange was found to contain maximum phenolics. The dried fractions were screened for their antioxidant activity potential using in vitro models such as DPPH, phosphomolybdenum method and by the nitroblue tetrazolium (NBT) reduction test at different concentrations. The MeOH : water (80:20) fraction of citron showed highest DPPH radical scavenging activity. All the fractions showed remarkable antioxidant capacity by the formation of phosphomolybdenum complex. In addition, all the extracts showed variable superoxide radical scavenging activity.

Conforti et al. (2007) showed the in vitro properties (antioxidant, hypoglycemic and anticholinesterase) of *C. medica* L. cv. Diamante. The n-hexane extract of Diamante citron peel was characterized by the presence of monoterpenes and
sesquiterpenes. The most abundant constituents were two monoterpenes, limonene and \( \gamma \)-terpinene. The extract showed significant antioxidant activity that was carried out using different assays, such as DPPH test, beta-carotene bleaching test and bovine brain peroxidation assay. Oxidative damage, caused by the action of free radicals, may initiate and promote the progression of a number of chronic diseases, including diabetes and Alzheimer’s disease. Diamante citron peel extract showed hypoglycemic activity and anticholinesterase effect.

**Belletti et al. (2007)** evaluated the combined effects of a mild heat treatment (55°C) and the presence of three aroma compounds [citron essential oil, citral and \((E)\)-2-hexenal] on the spoilage of noncarbonated beverages inoculated with different amounts of a *Saccharomyces cerevisiae* strain. The results, expressed as growth/no growth, were elaborated using a logistic regression in order to assess the probability of beverage spoilage as a function of thermal treatment length, concentration of flavoring agents and yeast inoculum. The logit models obtained for the three substances were extremely precise. The thermal treatment alone, even if prolonged for 20 minutes, was not able to prevent yeast growth. However, the presence of increasing concentrations of aroma compounds improved the stability of the products. The inhibiting effect of the compounds was enhanced by a prolonged thermal treatment. In fact, it influenced the vapor pressure of the molecules, which can easily interact within microbial membranes when they are in gaseous form. Citron gave the most interesting responses that is the beverages with 500 ppm of essential oil needed only 3 min of treatment to prevent yeast growth.

**Parveen & Latafat (2007)** carried out a study on 50 patients during 6-14 week of pregnancy to assess the efficacy of ‘Jawarish Anarain’, Unani medicine containing peel of *C. medica*, in vomiting. Overall improvement was observed in all the clinical signs and symptoms in patients.

**Monajemi et al. (2005)** have shown that *Citrus* essential oil contains different terpenes with antitumor activities. In this study researchers determined the cytotoxicity of essential oils of Iranian *Citrus limon* L., *C. medica* L., *C. sinsensis*
L. peels on cancer cell lines. The effects of *C. limon* (5-40 µg/ml), *C. medica* and *C. sinensis* (0.25-10 µg/ml) oil samples on two human tumor cell lines (MCF-7 and Hela) were determined. Different concentration of essential oils were added to cultured cells and incubated for 72 hour. The cell survival was evaluated using the MTT-based cytotoxicity assay. While limonene constitute about 98.4% and 98.8% in essential oils of *C. limon* and *C. sinensis*, respectively. Its’ percentage in *C. medica* was reported only 56.6%. In *C. medica*, there was a considerable amount of β-pinene, γ-terpinene, α-terpinolene and trans-α-bergamotene. IC₅₀ of essential oil for MCF-7 cell line was: *C. limon* ≈ 10 µg/ml, *C. medica* ≈ 1 µg/ml and *C. sinensis* ≈ 0.5 µg/ml. For Hela cell line IC₅₀ was: *C. limon* ≈ 17 µg/ml, *C. medica* ≈ 1 µg/ml and *C. sinensis* ≈ 3 µg/ml. This finding revealed that *C. limon* and *C. sinensis* had a greater cytotoxic effect on MCF-7 than that on Hela cells. These findings also indicated that *C. medica* and *C. sinensis* were more cytotoxic than *C. limon*. Comparison of the essential oil components of *C. limon* with those of *C. medica*, showed the presence of β-pinene (16.3%), α-terpineol (11.3%), γ-terpinene (4.4%) and trans- α-bergamotene (3.4%), which were not found in *C. limon*. Hence, it could be concluded that these components may have greater cytotoxic effects or they may also have synergistic effects with limonene.

**Feng et al. (2004)** reported that the constituents of *C. medica* var. *sarcodactylis* were isolated and purified by silica and Sephadex LH-20 gel column chromatography. Nine compounds were isolated and identified as 5-methoxy furfural (I), 5-hydroxy-2-hydroxymethyl-4H-pyran-4-one (II), diosmetin (III), diosmin (IV), obacunone (V), aviprin (VI), 3-(3-methoxy-4-hydroxyphenyl)-acrylic acid (VII), vanillic acid (VIII) and 3,4-dihydroxy-benzoic acid (IX). Compound I was a new natural compound. Compound II and IV-IX were isolated for the first time from the genus *Citrus* and compound III from *C. medica*.

**Silalahi (2002)** collected evidence from experimental and epidemiological studies and indicated that there was a low risk of degenerative diseases, cardiovascular disease, hypertension, cataract, stroke and in particular, cancers in people with a high intake of fruit and vegetables. This protective effect was assumed to be associated mainly with the antioxidant activities. The bioactive components
present in citrus fruits include vitamin C, β-carotene, flavonoids, limonoids, folic acid and dietary fibre. A high intake of citrus fruits may reduce the risk of degenerative diseases.

**Tian et al. (2001)** reported limonoids to inhibit the growth of estrogen receptor-negative and positive human breast cancer cells in culture. They studied the antiproliferative activity of four limonoids (obacunone-17, β-D-glucopyranoside, nomilinic acid-17,β-D-glucopyranoside, limonin, nomilin) and a limonoid glucoside mixture found in high concentrations in mandarin (*Citrus reticulata* Blanco), against a series of human cancer cell lines. The human cancer cell lines included leukemia (HL-60), ovary (SKOV-3), cervix (HeLa), stomach (NCI-SNU-1), liver (Hep G2) and breast (MCF-7). The growth-inhibitory effects of the four limonoids and the limonoid glucoside mixture against MCF-7 cells were found to be significant. The limonoids are reported in the seeds of citron.

**Govindachari et al. (2000)** tested antifungal activity of natural tetratriterpenoids such as limonin, limonol and nomilinic acid from *Citrus medica* against *Puccinia arachidis*, a groundnut rust pathogen.

**Divyajyot Ayurvedic Research Foundation Ahmedabad** reported effect of *Citrus medica* fruit in liver cancer. *Citrus medica* (bijoru) is a fruit used since ancient times by the Ayurvedic practitioners. In a research carried out in Divyajyot Ayurvedic Research Foundation, Ahmedabad, juice of a single bijoru fruit is given to the patients of primary as well as metastatic cancer registered at 'Divyajyot Ayurvedic research Foundation'. The study concluded that it improves patient's liver metabolism, gradually normalizes LFT, prevents recurrence and stops further spread of disease and decreases tumour growth. At the end of 4 to 6 months, patient becomes free from the disease symptoms and leads a normal life.

**Kretschmar & Baumann (1999)** studied the allocation of purine alkaloids within citrus flowers and found to be linked to anthesis, with 99% of the total flower caffeine confined to the androecium. The main alkaloid was caffeine accompanied by considerable (up to 30% of caffeine) concentrations of theophylline. In the anther, these purine alkaloids reach altogether a concentration
of 0.9% dry weight which is close to the caffeine content of the Arabica coffee bean. The pollen alkaloid concentration was found in the same range. Much lower but still marked concentrations were found in the nectar. A considerable breakdown of alkaloids during honey production was assumed.

**Mishra et al. (1992)** reported non-toxicity of *C. medica* leaves essential oil in rats. The authors studied the effect of chronic ingestion of a diet treated with different concentrations of essential oil of *C. medica* on body weight, diet consumption, haemoglobin, total and differential leucocyte count, blood glucose, protein, cholesterol, urea levels and glutamic oxalacetic transaminase, glutamic pyruvic transaminase and alkaline phosphatase activity in albino rats. It was concluded that the *C. medica* oil, which have been reported for their biological properties against storage fungi as herbal fumigant, is nontoxic to test animals and do not induce any adverse effects to the blood, liver function, kidney function, protein, carbohydrate and lipid metabolism of the animals.

**Raj (1975)** reported that alcoholic extract of *C. medica* rind showed moderate *in vitro* activity against human *Ascaris lumbricoides*.

**CHEMICAL CONSTITUENTS**

The chemical constituents reported in different parts of *C. medica* L. are mentioned below.

- The fruits are reported to contain flavonoids as hesperidin; 3,5,6-trihydroxy-4’,7-dimethoxyflavone;3,5,6-trihydroxy-3’,4’,7-trimethoxyflavone (Albach & Redman, 1969; Anonymous, 1992); vitamin C is also reported from fruits (Ajaiyeoba *et al.*, 2003).
- Seeds contain some tetrnortriterpenoids as limonin, limonol and nomilinic acid (Govindachari *et al.*, 2000). Limonin gives the intensely bitter taste to seeds.
- The pollens of its flowers are reported to contain purine alkaloids caffeine and theophylline (Kretschmar & Baumann, 1999).

The peel contains citrus flavonoids consisting of a mixture of hesperidoside (rhamnoglucoside of hesperetol), naringoside and ecyrodietyoside (flavanones). Essential oils and vitamin C are also present, in addition to glucosides herperidin and rutin (Andrews, 1961; Fleisher & Fleisher, 1991; Fleisher & Fleisher, 1996). The peel contains coumarins, limettin, scoparone, scopoletin and umbelliferone; besides nobiletin, limonin, diosmin, β-sitosterol and its β-D-glucoside (Mizuno et al., 1989).

Two new cyclic peptides were isolated from the fruit peels of *Citrus medica* var. *sarcodactylis* Swingle. Their structures were elucidated as cyclo (-Gly-Asp-Leu-Thr-Val-Tyr-Phe-) and cyclo (-Gly-Leu-Pro-Trp-Leu-Ile-Ala-Ala-) (Matsumoto et al., 2002).

**Peel essential oil**

Several studies have been performed on the chemical composition of the peel oil of citrons, although the literature data are not easily comparable because the ecological and extraction conditions were quite different (cold pressing, solvent extraction, steam distillation and hydrodistillation).

- The most abundant compounds of *C. medica* L. cv. Diamante peel oil were reported to be limonene, γ-terpinene, citral, geranial, β-pinene and α-pinene. A coumarin, citropten was also identified in *C. medica* L. cv. Diamante (Federica et al., 2010).

- Oil of variety ‘diamante’ from Calabria exhibited limonene (55-61%) and γ-terpinene (23-27%) as the main component (Capello et al., 1982; Cotroneo et al., 1986; Poiana et al., 1998).

- The composition of the oils of varieties ‘rugosa’ and ‘peretti’ exhibited a content of limonene (60-62%) similar to variety ‘diamante’, but differed by a lower amount of γ-terpinene (11-12%) and presence of β-pinene (14-15%) (Cotroneo et al., 1986).
In peel oil of the variety ‘corsican’ extracted with petroleum ether showed limonene (59%) and γ-terpinene (7%) as the major components. In another sample obtained by direct sampling in the oil gland, limonene (51%) was associated with geranial (13%) and neral (7%) (Huet, 1984; Huet, 1986).

The limonene, geranial, neral composition was also observed in peel oil of the variety ‘muliensis’ from china (Wen et al., 1986).

In peel oils of the variety ‘sarcodactylis’, four compositions may be distinguished: (a) limonene/γ-terpinene (48-56%/23-32%) (Shiota, 1990; Dung et al., 1996) (b) limonene/∆-3-carene (70%/15%) (Dung et al., 1996) (c) limonene/p-cymene (48%/34%) (Dung et al., 1996) and (d) neral/geranial/limonene (22%/19%/11%) (Zhu et al., 1993).

Peel oil of the variety ‘common ethrog’, exhibited a high proportion of limonene (81%) (Fleisher & Fleisher, 1991; Fleisher & Fleisher, 1996), while peel oil of the variety ‘yenemite ethrog’ was characterized by the limonene/γ-terpinene composition (53%/14%) (Fleisher & Fleisher, 1996).

A comparative study on chemical composition of peel oils of five citron varieties (ethrog, sarcodactylis, corsican, diamante and rhobs-el-arsta) had shown 36 components. Oil compositions differed only quantitatively. The major component in above five varieties was limonene (46.9-93.6%), associated with γ-terpinene (trace-31.3%), geranial (0.8-14.4%) and neral (0.4-7.6%) (Lota et al., 1999). The composition of the ‘rhobs-el-arsta’ oil exhibited a high amount of limonene (93.6%) and the contents of all other components were very low (≤ 1.5%), geranial and neral were accounted for 0.8% and 0.4%, respectively (Lota et al., 1999).

The oils of the corsican, sarcodactylis and ethrog varieties belonged to the limonene/γ-terpinene chemotype (46.9%-51.9%/ 26.2-31.3%), while contents of geranial and neral ranged from 1.3 to 5.4% (Lota et al., 1999).

Forty three components were reported in peel oil of C. medica L. grown in Bangladesh and identified as isolimonene (39.37%), citral (23.12%), limonene (21.78%), β-myrcene (2.7%), neryl acetate (2.51%) and neryl alcohol (2.25%) as major constituents (Bhuiyan et al., 2009).
Leaf essential oil

- The major components reported from leaf oil of ethrog variety are limonene (37.9%), geranial (8.2%), geraniol (6.2%), neral (5.1%), nerol (4.8%) and \((E)\)-caryophyllene (4.8%). The oil was obtained by hydrodistillation after maceration of leaves in alcohol (Fleisher & Fleisher, 1991; Fleisher & Fleisher, 1996).

- *C. medica* L. grown in Bangladesh reported to contain 19 components, dominated by erucylamide (28.43%), limonene (18.36%) and citral (12.95%). Erucylamide is the first reported component in *C. medica* L. leaf oil (Bhuiyan et al., 2009).

- In a study on leaf oil of five varieties (ethrog, sarcodactylis, corsican, diamante and rhobs-el-arsa) limonene was the major component (27.8-43.2%); other components were geranial (17.0-23.4%), neral (11.9-16.3%), geraniol (2.5-6.8%), nerol (2.1-7.3%) and their acetates, citronellal (0.6-2.1%), citronellol (0.7-1.4%) and linalool (1.1-1.6%). The oil of ‘rhobs-el-arsa’ citron exhibited a higher content of \((E)\)-\(\beta\)-ocimene and lower contents of nerol and geraniol (Lota et al., 1999).
REASONS FOR TAKING *CITRUS MEDICA* L.

The importance of *Citrus medica* L. is indicated from its traditional use in a number of ailments. The ethnopharmacological reports are mentioned for different parts of this plant as fruits, peels, seeds, roots, shoots, leaves and flowers. These plant parts are reported to be useful in many problems including sore throat, cough, asthma, thirst, typhoid fever, inflammation, intestinal worms, urinary calculus, vomiting, pain etc. Additionally it is used as a tonic, antiscorbutic, cardiotonic and sedative. In Kumaun region of Uttarakhand state, the fruit is widely consumed by the local people in winter season as ‘Sani-Nimu’ and as a pickle. Many varieties of *C. medica* L. are grown worldwide and most of the research work was done on varieties available in European countries, China and Japan.

To the best of our knowledge *C. medica* L. variety grown in Kumaun region is not evaluated for its biological activities. In some research works published in Indian Journals, the plant identification is not clear, in these articles *C. medica* L. is mentioned as lemon while lemon is now classified as *Citrus limon* (L.) Burm. f. and citron as *C. medica* L. So this creates confusion whether they worked on lemon or citron.

In literature, spring and rainy seasons are mentioned to be suitable for collection of leaves but no report is available regarding oil composition of *C. medica* L. leaves, collected during different seasons. So, the present work also includes oil analysis of leaves obtained during different seasons and grown at different places. Also no reports are available on essential oil analysis of *C. medica* L. flowers and roots, grown in Kumaun region.

OBJECTIVES AND PLAN OF WORK

Objectives

After detailed study of the plant and keeping in view of its importance it was thought worthwhile to carryout the systematic evaluation of *Citrus medica* L. The purpose of our study was to do pharmacognostic evaluation, evaluation of
pharmacological activity and phytochemical screening of extracts (solvent and steam distillate) of different parts of *Citrus medica* L. The parts of the plant selected were root, stem bark, leaves, flowers, fruit, fruit peel and seeds.

**Plan of work**

1. Comprehensive literature survey.
2. Authentication of the plant.
3. **Phytochemical analysis**
   - Collection of the plant materials when they are expected to have maximum activity.
   - Preparation of plant extracts.
   - Preliminary phytochemical investigation of plant extracts.
   - Extraction and chemical analysis of essential oils: it includes analysis of peel, root, flower oils and observation of seasonal and altitude variations on essential oil composition of leaf.
4. **Pharmacognostic evaluation**
   - Macroscopic characteristics.
   - Microscopic characteristics.
   - Determination of physicochemical parameters.
5. **Pharmacological evaluation**
   - It includes evaluation of following activities
     - Antioxidant activity of peel, pulp, juice and root.
     - Antimicrobial activity of root, leaf, bark, peel, pulp and juice.
     - Antiurolithiatic activity of fruit juice and root.
     - Analgesic activity of fruit decoction.
     - Anthelmintic activity of root and seed.
     - CNS activity of peel oil and distilled water of the fruit.
     - Hypolipidaemic activity of fruit juice in diabetic rats.
REFERENCES


