https://doi.org/10.56093/ijas.v90i6.104803

Indian Journal of Agricultural Sciences **90** (6): 1205–8, June 2020/Short Communication

Phenolic compounds from peel and callus extracts of sweet lime (*Citrus medica*)

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Received: 24 May 2019; Accepted: 04 September 2019

Key words: Antioxidant, Antibacterial, Citrus, Total phenolics, Total flavonoids

Citrus fruits, generally contain sugar, pectin, vitamins (A, B_1, and C), minerals, carotenoid pigments, organic acids such as citric acid and ascorbic acid and numerous active phytochemicals such as coumarins, and flavonoids as naringin, naringenin, hesperidin, neohesperidin, rutin, hesperetin, nairutin, and tangeretin. Sweet lime (*Citrus medica* L.) holds great importance in fruit kitchen garden. Although it has not gained much importance in solid block commercial plantation, it is preferred in fruit nutrition garden in backyards. It has also found its use in traditional medicine for cure of malaria and dengue. Some communities consume it as replacement of quinone-the drug used to cure malaria.

Further, fruits and vegetables are rich sources of health-promoting substances active in neutralization of reactive oxygen species (Arafa *et al*. 2015). Citrus peels are known to have high levels of phenolics which demonstrate strong antioxidant capability. The study represents callus cultures of plant as an interesting source for easy and scalable production of compounds which are thought to have antioxidant activities (Efferth 2019). The present investigation is aimed to observe the antioxidant activity of callus derived from different explants from *in vitro*-raised seedlings of sweet lime. The results obtained from the present study will be crucial for further understanding and development of plant to be used in treatment of diseases.

Plant material and surface sterilization of seeds: Mature fruits of sweet lime growing in citrus orchards of the Punjab Agricultural University, Ludhiana, were collected, washed, peeled; and peels were dried (45-50°C) and ground to fine powder. Callus was induced from different explants, i.e. epicotyl, hypocotyl and cotyledon of *in-vitro* raised seedlings in the Commercial Tissue Culture Laboratory of Punjab Agricultural University, Ludhiana during 2018-19. Seeds were extracted from ripened fruits and their outer seed-coat was removed with inner seed-coat intact. The seeds were washed with Bavistin followed by tap-water to remove fungal contamination. Under laminar air flow cabinet, the seeds were treated with mercuric chloride for 10 min followed by 3 washings with distilled water.

In-vitro raising, callus induction and subculturing: Surface-sterilized seeds were inoculated onto MS medium (Murashige and Skoog 1962) supplemented with 100 mg/litre myo-inositol and 30 g/litre sucrose and incubated at 24±2°C in 16:8 h light:dark conditions. Different explants (cotyledons, epicotyl, hypocotyl and leaves) excised from *in-vitro* raised seedlings were cultured on MS medium supplemented with 2 different concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D; 2.0 mg/litre and 4.0 mg/litre) and kinetin (Kin, 0.5 mg). Callus was induced under dark conditions under controlled temperature of 24 ± 2°C. Data on callus induction were recorded after 27 days of culture period.

Extractions of peel and callus in organic solvents: The peel and callus extracts of sweet lime were prepared using different concentrations (50, 80 and 90% *v/v*) of organic solvents, i.e. ethyl acetate (EA), isopropyl alcohol (IPA), methanol (ME) and ethanol (ET), using maceration, orbital shaking, ultrasound-assisted and soxhlet extractions. In maceration, powdered samples of peel and callus (10 g) were separately suspended in organic solvents (100 ml) for 72 h with stirring at intervals. In orbital shaking, samples of peel and callus (10 g) were shaken in solvents (100 ml) in orbital shaker for 24 h at 250 rpm. In ultrasonic-assisted extraction, callus and peel dissolved in solvents were placed in sonicator (40°C, 170 W, 42 KHz, 15 min). The extracts were prepared in different solvents by soxhlet extraction for 8-10 h. After giving respective treatments, all the extracts prepared were filtered, evaporated till dryness and stored in dark at 4°C till further use.

Estimation of total phenolic content and total flavonoid content of peel and callus extracts: Total phenolic content and total flavonoid contents of all extracts of peel and callus were estimated using Folin-Ciocalteu reagent (Ebrahimzadeh *et al.* 2008a, Ebrahimzadeh *et al*. 2008b) using gallic acid and quercetin as standard references respectively.

Antioxidant and antibacterial potential: The peel and callus extracts of sweet lime (obtained in higher yields and also found to contain higher TPC and TFC contents)

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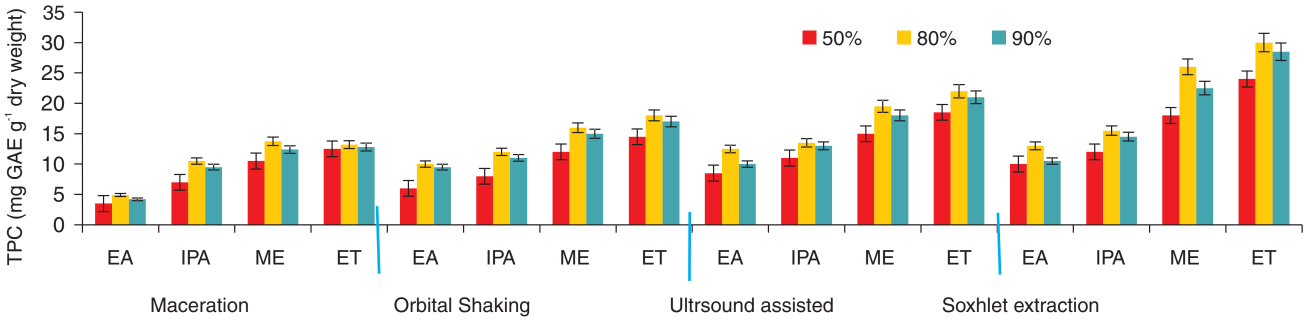

Fig 1 TPC of sweet lime peel extracts; EA-ethyl acetate, IPA-isopropyl alcohol, ME-methanol, ET-ethanol.

were evaluated in terms of their radical-scavenging ability using DPPH radical (Pan *et al*. 2006) using butylated hydroxyl toluene (BHT) as standard antioxidant. The inhibition percentage calculated was plotted as a function of concentrations of each test compound.

The extracts were also evaluated for their antibacterial activity against *Bacillus aryabhattai* (KF 853102) and *Klebsiella* sp. (KF 424316) by bacterial sensitivity-filter paper disc method (Davis and Stout 1971). Sterilized filter paper discs dipped in dimethyl sulfoxide served as control. Diameters of growth-inhibition zones (mm) were measured after 24 h. Extracts showing varying inhibition at different concentrations were evaluated for their minimum inhibitory concentrations (MIC) for test bacteria.

Maximum callus induction (100%) and fresh weight (3.5 g) were obtained from cotyledonary explants cultured on MS medium supplemented with 2, 4-D and Kin segments. Callus induction was not observed from leaves. Overall, the yields of peel extracts were higher than callus extracts, and Soxhlet extraction gave higher yields in all solvents, followed by ultrasonic extraction and orbital shaking. Among the solvents, ethanol extracts showed higher yields followed by methanol, isopropanol and ethyl acetate. 80% *(v/v)* of each solvent resulted in maximum yields of extracts among different concentrations tested, i.e.10.0, 12.5, 16.5 and 22.0% yield of peel extracts and 8.5, 11.0, 14.0 and 17.0% callus extracts for EA, IPA, ME, ET respectively, The yields of extracts decreased with the increasing concentration to 90% of each organic solvent. This may be attributed to degradation of phenolic compounds by highly concentrated solvents. These results support the findings of Safdar *et al.* (2017), who found that absolute solvents could not ensure fair extraction of polyphenols than aqueous solvents from kinnow (*Citrus reticulata* cv. *Kinnow Blanco*) peel. The yield of phenolic compounds from plants is associated with polarity, solubility, as well as certain extraction parameters such as nature of solvent, solvent concentration, extraction temperature, and time (Wang *et al*. 2008).

Soxhlet extracted methanol and ethanol extracts were found to have the highest TPC, i.e. 30 and 28.5 mg GAE/g dry weight, respectively (Fig 1). Peel extracts of sweet lime were found to have higher amounts of TPC and TFC than callus extracts. Also the major proportion of TPC was found to be TFC in all peel and callus extracts. DPPH radical scavenging activity in terms of percent inhibition of peel and callus extracts in 80% EA, IPA, ME and ET (100-1000 µg/ml) is presented in Fig 2. Peel extract in ET exhibited the maximum per cent inhibition. Callus extracts showed lesser per cent inhibition than peel extracts in respective solvents. Similarly, the per cent inhibition of DPPH radical of various extracts of *Stevia* leaves and callus were found to range between 33.17 and 56.82% (Tadhani *et al*. 2007). The antioxidant activities may be attributed to the phenolic and flavonoid contents, as it is reported that phenolic rings due to resonance stabilization of phenoxide ion contribute to antioxidant activities (Pan *et al*. 2006, Kaur and Uppal 2015).

Peel extract of sweet lime prepared in 80% ethanol by soxhlet extraction showed the highest antibacterial potential against *Bacillus aryabhattai* and *Klebsiella* sp. (Table 1 and 2). Minimum inhibitory concentration of ethanol extract was 20 and 25 µg/disc against *Bacillus aryabhattai* and *Klebsiella* sp. respectively. Similar observations were recorded by Johnson *et al.* (2011) on antibacterial activity

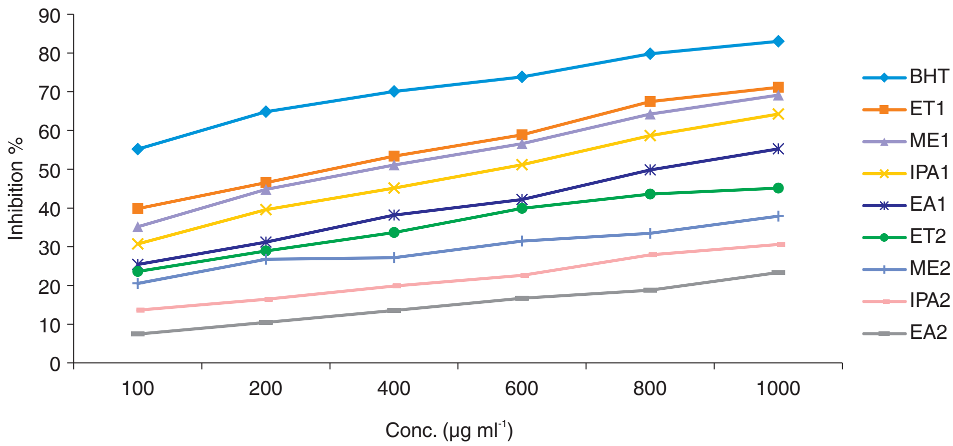

Fig 2 Inhibition percentage of peel and callus extracts of sweet lime in 80% *(v/v)* of respective solvents; EA-ethyl acetate, IPA-isopropyl alcohol, ME-methanol, ET-ethanol, BHT- butylated hydroxytoluene; 1 and 2 stand for peel and callus extracts, respectively.

Table 1 Antibacterial activity (µg/disc) of peel extracts of *Citrus medica* at different concentrations prepared by soxhlet extraction in 80% (v/v) of respective solvents

Extract	Diameter of growth inhibition zone (mm)						
	3000	2000	1000	500	100	50	MIC (µg/disc)
Bacillus aryabhattai							
EA	11.05±1.0	9.25±0.2	7.50±1.0	6.52±0.5	6.14±1.0	6.00±0.0	85
IPA	13.52±0.7	10.50±0.4	9.05±1.0	7.45±1.0	6.52±0.5	6.00±0.0	70
ME	17.35±0.5	14.60±1.0	12.52±0.5	9.54±0.5	7.75±0.4	6.50±0.2	40
ET	18.15±0.5	16.52±0.5	14.70±0.2	11.20±0.2	9.51±0.5	7.04±0.5	20
Klebsiella sp.							
EA	9.52±1.0	7.42±0.4	6.54±0.5	6.25±0.5	6.00±0.0	6.00±0.0	175
IPA	10.45±0.5	8.51±0.5	7.10±0.2	6.54±1.0	6.12±0.8	6.00±0.0	80
ME	12.05±0.4	10.02±0.8	9.45±0.5	8.17±0.5	7.25±0.2	6.52±0.2	30
ET	13.52±0.5	12.20±1.0	10.52±0.5	9.25±0.8	8.05±0.5	7.15±0.8	25
Tetracycline	28.15±0.5	25.54±1.0	19.57±1.0	16.82±0.5	14.52±1.0	12.80±0.5	-

Table 2 Antibacterial activity (µg/disc) of peel extracts of *Citrus medica* at different concentrations prepared by soxhlet extraction in 80% (v/v) of respective solvents

Extracts	Diameter of growth inhibition zone (mm)						
	3000	2000	1000	500	100	50	MIC (µg/disc)
Bacillus aryabhattai							
EA	8.45±0.2	7.55±0.7	6.25±0.6	6.12±0.5	6.00±0.0	6.00±0.0	350
IPA	8.90±0.5	7.85±0.6	6.87±0.5	6.25±0.7	6.10±0.5	6.00±0.0	95
ME	9.20±1.0	8.65±0.5	7.56±0.7	6.75±0.1	6.50±0.4	6.00±0.0	80
ET	9.50±0.8	8.85±0.4	7.95±0.5	6.90±0.2	6.65±0.3	6.25±0.5	65
Klebsiella sp.							
EA	6.75±0.5	6.25±0.4	6.00±0.5	6.00±0.0	6.00±0.0	6.00±0.0	1500
IPA	7.05±0.1	6.55±0.5	6.25±0.4	6.00±0.0	6.00±0.0	6.00±0.0	850
ME	8.85±0.2	8.25±0.8	7.75±0.2	6.35±0.2	6.00±0.0	6.00±0.0	350
ET	9.50±0.1	8.55±0.1	7.85±0.2	6.50±0.4	6.00±0.0	6.00±0.0	180
Tetracycline	22.15±1.0	20.55±1.0	17.50±0.1	15.50±0.2	13.60±1.0	10.50±0.4	-

of leaves and inter-nodal callus extracts of *Mentha arvensis,* where the ethanol extracts of leaves derived calli showed maximum bio-efficacy than other solvents.

The peel and callus extracts of sweet lime may, therefore, be recommended for use in foods to prevent oxidation of fats and oils and as antibacterial. Our results indicated that sweet lime fruit and callus culture-based antioxidants may also be further exploited for applications such as nutraceutical and preservation of food products as callus cultures can be maintained throughout the year in the laboratory as compared to sweet lime fruits available in particular fruit season.

SUMMARY

An experiment was conducted to quantify and evaluate bioactivity of phenolics from peel and callus extracts of sweet lime (*Citrus medica*). Cotyledon, epicotyl and hypocotyl-derived calli of sweet lime were obtained on solid MS medium supplemented with 2, 4-D and Kin. Cotyledon exhibited the best response on callus induction medium. Various extracts were prepared from peel and callus using different solvents, i.e. 50, 80 and 90% (*v/v*) of ethyl acetate, isopropyl alcohol, methanol and ethanol, employing maceration, orbital shaking, ultrasonic assisted and soxhlet extractions followed by estimation of total phenolic content (TPC) and total flavonoid content (TFC). Soxhlet extraction with 80% ethanol from peel was found to be the best combination as the yields of extracts; TPC and TFC were found to be the highest. Peel extract of sweet lime (80% ethanol, soxhlet) showed the highest antioxidant potential, i.e. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and antibacterial potential against *Bacillus aryabhattai* and *Klebsiella* sp. Thus, peel of sweet lime, being a potential source of phenolic compounds with antioxidant and antimicrobial properties, may be used as an ingredient for the preparation of functional foods.

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