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ANTIMICROBIAL SCREENING AND PHYTOCHEMICAL ANALYSIS OF *CITRUS* CULTIVARS GROWING *IN VIVO* & *IN VITRO*

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Citrus fruits are potential source of medicinal importance from archaic period. Antimicrobial resistance and side effects of antibiotics is the most common worldwide problem. To minimize these problems, search for novel antimicrobials from natural resources has been increased. The present study was carried out to find out bioactive potential of Citrus cultivars growing in*vivo* and *invitro.* Leaves, peel, pulp & tissue culture extracts prepared in benzene, ethanol and methanol were screened against four Gram positive, four Gram negative bacteria and two fungal pathogen using agar well diffusion method. Extracts were further analyzed for the minimum inhibitory concentration (MIC).Phytochemical analysis of active extracts showed the presence of alkaloids, glycosides, flavonoids, saponins, steroids, and tannins.

Key words: Antimicrobial screening, Citrus plants**,** Minimum inhibitory concentration, Phytochemicals

Introduction

Medicinal plants are the natural resources in developing of new drugs.[1] Nature has been a source of medicinal agent for thousands of years and an impressive number of modern drugs have been isolated from natural sources, based on their use in traditional medicine[2]. This is due to increased source awareness of the limited ability of the synthetic pharmaceutical products to control major diseases. The basic molecular and active structures for synthetic fields are provided by rich natural sources[3].

The genus Citrus, belonging to the Rutaceae family, comprise of about 140 genera and 1,300 species. Citrus fruits are suggested as antimicrobial agents from ancient times. The Citrus fruits and their by–products are of high economic and medicinal value because of their multiple uses, such as in food industry, cosmetics and folk medicine[4]. The citrus plants are easily available, cheap, less side effecters as compared to allopathic & chemical drug. Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities[5, 6]. In addition, the fibre of citrus fruit also contains bioactive compounds, such as polyphenols, the most important being vitamin C (or ascorbic acid), and they certainly prevent and cure vitamin C deficiency-the cause of scurvy[7].The peel of *Citrus* fruit is a rich source of flavanones and many polymethoxylated flavones, which are very rare in other plants[8]**.** In additions to large scale consumption as fresh fruits, the fruits are mainly processed to produce juice. The waste of Citrus processing industry left after juice extraction, such as peels, seeds and

pulps, corresponding to about 50% of the raw processed fruit, can be used as a potential source of valuable by products[9]. Specifically, the Citrus peels, commonly treated as agro-industrial waste, are a potential source of valuable secondary plant metabolites and essential oils[10].

In the present investigation extracts of different plant parts and unorganized callus of two Citrus cultivars viz.*Citrus nobilis* (Kinnow) and *C. sinensis* (Malta) were prepared in different solvents (Benzene, Ethanol and Methanol) and tested against four Gram positive, four Gram negative and two fungal strains using agar well diffusion method. The extracts were also analyzed for the minimum inhibitory concentration and major phytoconstituents.

Material and Methods

The authentic plant material of Citrus cultivars - Kinnow and Malta was procured from Agriculture Research Station, Sriganganagar and Bikaner (Rajasthan), India respectively.

Establishment of *in vitro* cultures:

Unorganized tissue cultures of Citrus plantswere established from the leaf explant using Murashige and Skoog's[11] medium supplemented with 1.5 mg/L 2,4-D and 0.5 mg/L kinetin and maintained for 10-12 months by frequent subculturings at intervals of 4-6 weeks. The growth indices were calculated at 2, 4, 6, 8 and 10 weeks. Callus tissues at the transfer stages of the maximum growth index were then used for antimicrobial screening.

Extraction and preparation of test samples:

The Leaves, peel, pulp and callus tissues dried, homogenized into fine powder were subjected to Soxhlet extraction in benzene, ethanol & methanol for 24 hrs and allowed to concentrate in vacuum at room temperature.

Test microorganisms:

The test microorganisms include four Gram positive bacteria (*Bacillus cereus* NCIM 2156, *Staphylococcus aureus* NCIM 2654, *S. epidermidis* NCIM 2493, *Mycobacterium smegmatis* NCIM 5138), four Gram-negative bacteria (*Escherichia coli* NCIM 2685, *Pseudomonas aeruginosa*NCIM 5032, *Proteus vulgaris* NCIM 2027, *Salmonella typhimurium*NCIM 2501) and two fungal pathogens *Candida albicans*(NCIM 3466) and *Trichodermaviride*(NCIM 1221) which were obtained from the National Chemical Laboratories (NCL), Pune (India) for the present investigation.

Inoculum preparation:

The culture medium used for *B. cereus, S.aureus, S. epidermidis, P.aeruginusa, P. vulgaris,S. typhimurium, E. coli,* was nutrient broth (0.5 % peptone, 0.3 % yeast extract and 0.3 % NaCl pH adjusted to 7) whereas, in case of *M. smegmatis*, Mpheli medium (0.5 % KH2PO4, 0.006 % MgSO4,0.25 % sodium citrate, 2 % glycerol and 0.5 % asparagine pH adjusted to 7.8) was used. To culture *T.viride*, Sabouraud's liquid medium (0.1 % peptone and 0.4 % dextrose pH adjusted to 5.6) was used and for *C. albicans*, MGYP liquid medium (0.3 % malt extract, 1 % glucose, 0.3 % yeast extract, 0.5 % peptone, pH 6.4-6.8) was used. The test organisms maintained on agar slants were recovered for testing by inoculating in the respective broth and incubating at 37 ºC (in the case of bacteria) and 28 ºC (in the case of fungi) in a shaker at 180 rpm until the concentration of the test organisms reached that of the 0.5 McFarland standard[12].

Antimicrobial assay:

The agar well diffusion method[13] was adopted for the antimicrobial screening. Wells 8 mm in diameter were punched into the agar medium and filled with 100 µl plant extract (200 mg/ml), solvent blanks and standard antibiotics (positive controls). The plates were then incubated at 37°C for 18 – 24 hrs and antimicrobial activity was evaluated by measuring the observed inhibition zone diameter. Each experiment was performed in five replicates.

Reference antibiotics:

Reference antibiotics Chloramphenicol (25µg) was used for Gram-positive bacteria (*B. cereus, S. aureus, and S. epidermidis*) and Gram-negative bacteria (*E. coli, P. aeruginosa, P. vulgaris* and *S. typhimurium*); Streptomycin (25µg) was used for *M.smegmatis,* Amphotericin B (25µg) and Fluconazole (25µg) for fungi *C. albicans*and *T.viride* respectively were used as references for a comparison of the antimicrobial activity of the test samples.

Minimum Inhibitory Concentration (MIC):

Microdilution broth susceptibility assay[14] with some modifications was used for the determination of the minimum inhibitory concentration of active plant extracts. A sterile 16 well plate was labeled. 100 µL of nutrient broth was added to all the wells. A volume of 100 µL of test material in 10% (v/v) DMSO or sterile water (usually a stock concentration of 100 mg/mL was pipetted into the first row of the plate. Serial dilutions were performed using a micropipette to obtain dilutions: 50mg/ml, 25mg/ml, 12.5mg/ml, and finally 6.25mg/ml. 10 µL of bacterial suspension was added to each well. Negative and positive control was set up for each test organism. The plates were prepared in duplicate, and placed in an incubator set at 37 °C for 18–24 h. Growth of the microorganisms was determined by taking absorbance at 620nm on an automated microplate reader (Spectra Max and Spectrophotometer).The lowest concentration of an extract that completely inhibit the growth of the microorganism was taken to represent the minimum inhibitory concentration of the test sample and was expressed in µg /ml. The average of two values was calculated and that was the MIC for the test material and bacterial strain.

Phytochemical screening:

Phytochemical screening of active extracts was carried following the standard qualitative methods as described by various researchers[15-16] to detect for the presence of biologically active compounds like alkaloids, glycosides, flavonoids, saponins, steroids and tannins.

Results and Discussion

The unorganized callus of both cultivars was fragile and pale yellow in colour. Maximum GI was observed at eight weeks. Ethanol extract of dried peel of *Citrus sinensis* evaluated for antimicrobial activity against some medically important bacteria *Staphylococcus aureus, Escherichia coli, Pseudomonas aerogenes , Bacillus cereus*) and fungi (*C. albicans*) showed antibacterial and antifungal effect against these pathogens[17] however the results in Table 1 shows that the same extract of leaves, peel and pulp exhibited remarkable inhibitory effect against not only these pathogens but also against *S.epidermidis, M.smegmatis , P.vulgaris, S.typhi and T.viride.* Benzene extract of Peel and pulp of Kinnow exhibited antibacterial activity against all the bacterial species tested and leaves extract was found to be active against *M. smegmatis, S.typhi and P. aeruginosa* but they did not show inhibitory effect against fungal species tested however Benzene extract of leaves, peel and pulp of Malta displayed antimicrobial effect against *B. cereus, S. typhi, P. aeruginosa and C. albicans* while they were found to be inactive against other microorganisms tested.

Methanolic extract of leaves and peel of Kinnow showed antimicrobial activity against *B. cereus, S.aureus, S.epidermidis, E.coli, P.vulgaris, S.typhi, P. aeruginosa and C.albican*s whereas pulp extract exhibited activity *against P.vulgaris S.typhi, S.aureus, P. aeruginosa and C.albican*s. Similarly in case of Malta methanolic extract of leaves showed activity against all the microbial strains but found to be inactive against *S.typhi* and *T.viride* whereas peel extracts showed activity against all the bacterial and fungal species except *T.viride.*

Microorganisms	*Citrus nobilis* (Kinnow)												*Citrus sinensis* (Malta)											
	Leaves			Peel			Pulp			*In vitro* callus			Leaves			Peel			Pulp			*In vitro* callus		
	BE	ET	ME	BE	ET	ME	BE	ET	ME	BE	ET	ME	BE	ET	ME	BE	ET	ME	BE	ET	ME	BE	ET	ME
B. cereus	--	0.90	0.36	0.54	1.18	0.72	0.36	0.95	--	--	0.50	--	0.45	0.72	0.40	0.36	0.81	0.36	0.40	22	--	--	0.40	--
S. aureus	--	0.45	0.29	0.29	0.58	0.33	0.33	0.66	0.37	--	0.33	--	--	0.37	0.50	--	0.75	0.41	--	0.75	--	--	0.29	--
S.epidermidis	--	1.04	0.47	--	0.71	0.66	--	0.80	--	--	0.57	--	--	0.52	0.57	--	0.76	0.57	--	0.95	--	--	0.52	--
M. smegmatis	0.38	0.44	--	0.44	0.38	--	0.44	1.0	--	--	0.50	--	--	0.55	0.61	--	1.11	0.44	--	0.94	--	--	0.50	--
P. vulgaris	--	0.94	0.47	0.42	0.57	0.42	0.42	0.63	0.52	--	0.36	--	--	0.68	0.47	--	0.63	0.52	--	0.68	--	--	0.42	--
E. coli	--	0.94	0.64	0.94	0.47	0.70	19	0.70	--	--	0.58	--	--	0.47	0.58	--	1.0	0.47	--	1.17	0.58	--	0.64	--
S.typhimurium	0.40	0.50	0.45	0.45	0.81	0.36	0.68	0.90	0.40	--	0.36	--	0.36	0.40	--	0.31	0.81	0.50	0.36	0.63	0.40	--	0.36	--
P. aeruginosa	0.45	0.40	0.35	0.40	0.95	0.40	0.45	0.70	0.55	--	0.55	--	0.35	0.55	0.40	--	0.80	0.45	0.40	0.95	0.50	--	0.40	--
C. albicans	--	0.38	0.42	--	0.80	0.38	--	0.71	0.52	--	0.38	--	0.52	0.42	0.57	0.42	0.71	0.42	0.33	0.80	0.38	--	0.38	--
T. viride	--	0.37	--	--	0.24	--	--	0.79	--	--	0.29	--	--	0.37	--	--	0.54	--	--	0.50	--	--	0.29	--

Table – 1: Antimicrobial Screening (Activity Index) of Citrus plants

BE= Benzene, ET=Ethanol, ME- Methanol

Activity index= Ratio of diameter of inhibition zone due to Plant part extract under observation and diameter of inhibition zone due to standard reference antibiotics
Average inhibition zone:Streptomycin (30 μg) against *B.cerus* = 22 mm; *S.aureus* =24 mm; *S.epidermidis*=21 mm; *M.smegmatis*= 18 mm. Ampicillin (30 μg) against *E.coli*= 17 mm; *P.aeruginosa*= 20 mm; *P.vulgaris*=19 mm; *S.typhimurium*= 22mm; Amphotericin B (30 μg) against *C.albicans*= 21 mm; Fluconazole(30 μg) against *T.viride*= 24

Microorganisms	*Citrus nobilis* (Kinnow)												*Citrus sinensis* (Malta)											
	Leaves			Peel			Pulp			*In vitro* callus			Leaves			Peel			Pulp			*In vitro* callus		
	BE	ET	ME	BE	ET	ME	BE	ET	ME	BE	ET	ME	BE	ET	ME	BE	ET	ME	BE	ET	ME	BE	ET	ME
B. cereus	--	20	>100	40	18	30	>100	20	--	--	50	--	>100	40	>100	>100	40	>100	>100	20	--	--	>100	--
S. aureus	--	50	>100	>100	40	>100	>100	35	>100	--	>100	--	--	>100	50	--	40	50	--	30	--	--	>100	--
S.epidermidis	--	18	>100	--	30	30	--	40	--	--	55	--	--	50	55	--	40	55	--	20	--	--	50	--
M. smegmatis	>100	>100	--	>100	>100	--	>100	30	--	--	>100	--	--	55	50	--	20	>100	--	30	--	--	>100	--
P. vulgaris	--	40	>100	>100	50	>100	>100	50	>100	--	>100	--	--	55	>100	--	50	50	--	55	--	--	>100	--
E. coli	--	30	50	30	>100	>100	25	50	--	--	55	--	--	>100	55	--	40	>100	--	30	>100	--	55	--
S.typhimurium	>100	50	>100	>100	25	>100	40	20	>100	--	>100	--	>100	>100	--	>100	40	55	>100	40	>100	--	>100	--
P. aeruginosa	>100	>100	>100	>100	25	>100	>100	60	50	--	50	--	>100	50	>100	--	40	>100	>100	30	55	--	>100	--
C. albicans	--	>100	>100	--	30	>100	--	40	>100	--	>100	--	55	>100	60	>100	40	>100	>100	60	>100	--	>100	--
T. viride	--	>100	--	--	30	--	--	25	--	--	>100	--	--	>100	--	--	55	--	--	55	--	--	>100	--

Table – 2: Minimum Inhibitory Concentrations (µg/ mL)
BE= Benzene, ET=Ethanol, ME- Methanol

Plant	Plant part extract	Phytoconstituents					
		Alkaloids	Cardiac glycosides	Flavonoids	Steroids	Saponins	Tannins
Citrus nobilis **(Kinnow)**	Leaves	+	+	++	+	-	+
	Peel	+	+	+++	+	+	+
	Pulp	+	+	+++	+	-	+
Citrus sinensis **(Malta)**	Leaves	+	+	++	+	-	+
	Peel	+	+	++	+	-	+
	Pulp	+	+	+++	+	+	+

Table- 3 Phytochemical analysis of plant extracts

Pulp extract showed activity against *E.coli, S.typhi, P. aeruginosa* and *C.albicans* only. The benzene and methanolic extracts of unorganized callus of both the plant cultivar did not show antimicrobial activity against any of the microorganism tested. Cowan[18]stated that the antimicrobial property of citrus peel depends on the type of solvent used for extraction especially due to its constituents of aromatic and organic antibiotic compounds of plant which are easily soluble in organic solvent. Antibacterial effects of various citrus peels have been demonstrated in the literature [19- 20] .The ethyl acetate extracts of the citrus peels exhibited inhibitory effect against food borne bacteria[22]. Antimicrobial activity of citrus fruit pulp, whole citrus fruit juice and silver nanoparticles synthesized using citrus fruit pulp against Gram positive, Gram negative bacteria and fung was reported by Suneeta et al[23].

Tissue cultures have also been reported to have antimicrobial activity. In the present study the ethanolic extracts of unorganized callus of both the plants also showed significant antimicrobial effect against these microorganisms. Methanolic extract of leaf derived callus of *Datura stramonium L.* showed activity against *Bacillus subtilus and Candida albicans*[24.] Aquous extract of *Clitoria ternatea* callus found to be active against *S.aureus, E.coli and S.typhimurium*[25].

The extracts showed inhibitory effect were analyzed to determine minimum inhibitory concentration against the bacterial and fungal species (Table-2). The MIC 18 µg $mL^{-1,}$ 25 µg mL^{-1}& 30 µg mL^{-1} was observed against *B.cerus, P. aeruginosa* and *C.albicans* in ethanolic extract of Peel, 18 µg $mL^{-1,}$& 40 µg $mL^{-1,}$ against *S. epidermidis and P.vulgaris* in ethanolic extract of leaves: 20 µg $mL^{-1,}$ & 25 µg $mL^{-1,}$ against *S. typhimurium, and T.viride* respectively in ethanolic extract of pulp and 25 µg mL^{-1} against *E. coli* in the benzene extract of pulp of Kinnow however MIC 30 µg mL^{-1} & 20 µg mL^{-1} was observed against *S. aureus and M.smegmatis* in ethanolic extract of pulp and peel of Malta respectively. The peel extracts of Kagja Lemon, South African Malta, and Dargiling Orange showed antibacterial activity on *B. cereus* with inhibitory concentration 31.25 µg/ml[26]. Johan et al[27] found hexane extract of various citrus peels has MIC value between 500-2000 µg/ml on *S. aureus.* The test extracts showed mild to broad spectrum activity against one or more test microorganism which indicates the presence of broad spectrum antimicrobial compound.

Phytochemical analysis of active extracts showed the presence of common phytoconstituents like alkaloids, flavonoids, glycosides, steroids, saponins and tannins (Table-3). Mamta and Parminder[28] demonstrated the presence of tannins, saponins in both the citrus peel and pulp. Generally, phytochemicals are known to have many health benefits such as anti-inflammatory, antimicrobial, antihypertensive, and antidiabetic effects[29-30].The presence of flavonoids, alkaloids, steroids, terpenoids, saponins, cardiac glycosides, and reducing sugars in all the juice concentrates studied affirming that citrus fruits are rich sources of phytochemicals[31].

Conclusion

The results of the present study indicate that extracts of these plant *invivo* and *invitro* possess significant antimicrobial activity against a number of bacterial and fungal strains of clinical significance and this confirms the value of the plant as a traditional medicine. The results of this study have revealed that these commonly consumed citrus fruits may contain promising antimicrobial leads. The investigation also indicates that tissue culture techniques are quite promising.

Further studies of the active plant extracts are needed involving the pharmacological evaluation and isolation of potentially therapeutic bioactive antimicrobial agents.

References

1. Shakya AK, Sharma N, Saxena M, Shrivastava S and Shukla S 2012, Evaluation of the antioxidant and hepatoprotective effect of Majoon-e-Dabeed-ul-ward against carbon tetrachloride induced liver injury. *Experiment Toxicology and Pathology*, 64 **7- 8** 767- 773.
2. Chaudhari TB, Tambe DA and Chaudhari SR. 2012, Phytopharmacology of Tephrosiapurpurea Pers. (Fabaceae) A Review. *J of Pharmacog and Herb Formul*,2 **8** 1-13
3. Preeti R, Vimal L, Devanathan and Loganathan M 2010, Antimicrobial and antioxidant efficacy of some medicinal plants against food borne pathogens. *Adv. Biol. Res* **2** 122-125.
4. Silalahi J 2002, Anticancer and health protective properties of Citrus fruit components. *Asia Pac J ClinNutr* **11** 79-84.
5. Amita T, Mridula M and Pragya R 2012, Pharmacological importance of citrus fruit. *International journal of pharmaceutical science and research.***4** 156-160.
6. Amiya KP and Prasanna P 2014, Green Synthesis of Silver Nanoparticle Using *Eichhornia crassipes* and Study of *in-vitro* Antimicrobial Activity. *Scholars Academic Journal of Pharmacy*, 3 **6** 504-509.
7. Donatus EO 2008, Citrus fruits: a rich source of phytochemicals and their roles in human health. *Int. J. Chem. Sci.* 6 **2** 451-471.
8. Ahmad MM, Salim-ur-Rehman ZI, Anjum FM and Sultan JI 2006, Genetic variability to essential oil composition in four citrus fruit species. *Pak. J. Bot*. 38 **2** 319-324.
9. El-adawy TA, Rehman EH, El-Bedaway AA and Gafar AM 1999, Properties of some Citrus seeds. Part 3. Evalaution as a new source of Potential oil. *Nahrung*, **43** 385-391.
10. Andrea V, Nadia N, Teresa RM and Andrea A 2003, Analysis of some Italian Lemon liquors (Limoncello). *J Agric Food Chem.***51** 4978-4983.
11. Murashige T and Skoog F 1962, A revised medium for rapid growth and bioassays with Tobacco tissue culture. *Physiologia Planetarium*,**15** 473- 479.
12. Prashanth KV, Neelam S, Chauhan HP and Rajani M 2006, Search for antibacterial and antifungal agents from selected Indian medicinal plants. *J Ethnopharmacol*, **107** 182-188.
13. Perez C, Pauli M and Bazerque P 1990, An antibiotic assay by the agar well diffusion method. *ActaBiologiaeet Medicine Experimentalis*,**15** 113–115.
14. Zgoda JR and Porter JR 2001. A convenient microdilution method for screening Natural products against bacteria and fungi. *Pharm. Biol.* 39 **3** 221-225.
15. Fadeyi MQ, Adeoye AE and Olowokudejo JD 1989, Epidermal and phytochemical studies in the genus *Boerhavia*(Nyctanginaceae) in Nigeria. *International Journal of Crude Drug Research* **29** 178–184.
16. Odebiyi A and Sofowora AE 1990, Phytochemical screening of Nigerian medicinal plants. Part III. *Lloydia* **41** 234–246.
17. Rehab MAE, Areej SBS and Heelah FA 2018, The phytochemical and antimicrobial effect of Citrus sinensis (Orange) peel powder extracts on some animal pathogens as Eco-friendly. EC Microbiology, 14 **6** 312-318.
18. Cowan M 1999, Plant products as

antimicrobial agents. *Clinical Microbiology Reviews* 12 **4** 564-582.
19. Lawal D, Bala JA, Aliyu SY and Huguma MA 2013, Phytochemical Screening and *In Vitro* Anti-Bacterial Studies of the Ethanolic Extract of *Citrus Senensis* (Linn.) Peel against some Clinical Bacterial Isolates. *Int J of Innov and Appl Stud*, 2 **2** 138-145.
20. Ramachandra YL, Ashajyothi C, Sreepadaraja HJ and Padmalatha RS 2013, Antimicrobial activity of Citrus rind oil isolated from *Citrus aurantium. Int J of Pharma World Res* 4 **1** 79-84.
21. Dhanavade MJ, Jalkute BC, Ghosh SJ and Sonawane DK 2011, Study Antimicrobial Activity of Lemon (Citrus lemon L.) Peel Extract. *Br J Pharmacol&Toxicol* 2 **3** 119- 122.
22. Chanthaphon S, Chanthachum S and Hongpattarakere T 2008, Antimicrobial activities of essential oils and crude extracts from tropical Citrus spp. against food related microorganisms. *Song J of Sci and Technol*, **30 1** 125-131.
23. Suneeta P, Pawan C, Amit S, Laxmi S and Nivedita K 2017, Antimicrobial Activity of Citrus Fruit Pulp, Whole Citrus Fruit Juice and Silver Nano Particles Synthesized Using Citrus Fruit Pulp. *Asian Journal of Pharmaceutical Technology & Innovation*, 5 **22** 06 -15.
24. Mawahib E, Zaydan F, Abd-Al-Rahman M and Yagoub SO 2014, Callus induction and antimicrobial activities of callus and intact plant extracts of *Datura stramonium* L. International Journal of Science and Research. 358 **8** 1105-1109.
25. Mhaskar AV, Prakash K, Vishwakarma, K S and Maheshwari VL 2010, Callus Induction and antimicrobial activity of seed and callus extracts of *Clitoria ternatea*L. Current Trends in Biotechnology and Pharmacy. 3 **4** 561-567.
26. Shyla A, Faysun NF, Mushkika J, Khokon MA, Sanjia Mehjabin, GM and Masud Parvez 2017, Antibacterial Activity of Different Citrus Fruits Specialty *Journal of Medical research and Health Science*, 2 **2** 25-32.
27. Johan S, Oliveira VL, Pizzolatti MG, Schripsema J, Braz-Filho R, Branco A and Smânia A 2007, Antimicrobial activity of wax and hexane extracts from Citrus Spp. Peels. *Mem Inst Oswaldo Cruz*, 102 **6** 681-685.
28. Mamta Arora and Parminder Kaur 2013, Phytochemical screening of orange peel and pulp. *International Journal of Research in Engineering and Technology*, 2 **12** 517-522.
29. Ayoola GA, Folawewo AD, Adesegun SA, Abioro OO, Adepoju-Bello AA and Coker HAB 2008, Phytochemical and antioxidant screening of some plants of Apocynaceae from South West Nigeria. *Afr. J. Plant Sci.* **2** 124–128.
30. Oikeh EI, Oriakhi K, and Omoregie ES 2013, Proximate analysis and phytochemical screening *of Citrus sinensis*fruit wastes. *Bioscientist* **1**164–170.
31. Mathew BB, Jatawa SK and Tiwaari A 2012, Phytochemical analysis of *Citrus limonum* pulp and peel. *Int. J. Pharm. Pharm. Sci*. **4** 269–371.