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Vitamin C Determination and Isolation in *Phyllanthus emblica*, *Citrus aurantium* and *Citrus limon* Peels by RP-HPLC Method

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ABSTRACT

Flavonoid Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods. The most commonly used Synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Propyl gallate (PG), and butylated hydroquinone. However, these synthetic antioxidants have side effects such as liver damage and carcinogenesis. Therefore, there is a need for isolation and characterization of natural antioxidant having less or no side effects, for use in foods or medicinal materials in order to replace synthetic antioxidants. The importance of aromatic plants as natural antioxidants has been well established. Flavonoid antioxidant (Vitamin C) present in *Emblica officinalis* linn, is known for its biological activities such as antioxidant, antidiabetic, antibacterial, antistress, anti-inflammatory, ophthalmopathy, natural preservative, anticancer, antitussive, antiepileptic, antipyretic, hepatoprotective, nephroprotective, and anti-anaemic, immune modulators. Also present in *Aleuria aurantia* having biological activities of antioxidant, anti-inflammatory, anticancer, antimicrobial, antidiabetic, and used to treat cardiovascular diseases, asthma, and decrease cholesterol.

INTRODUCTION:

The therapeutic efficacy of many indigenous plants, for various diseases, has been described by traditional herbal medicinal practitioners. Natural products are the source of synthetic traditional herbal medicine. They are still the primary health care system in some parts of world. In India, local imperial knowledge about medicinal properties of plants is the basis for their uses as a home remedies. It is generally accepted by many Indians and elsewhere in the world that beneficial medicinal effects can be obtained by ingesting plant products. Plants have bear the basis of many traditional medicines throughout the word for thousands of years and continue to provide new remedies to mankind. *Phyllanthus emblica* obtained from family Euphorbiaceae commonly known as amla, aleuria aurantia family rutaceae commonly known as orange peel and *Citrus limon* obtained family rutaceae commonly known as lemon peel. The peels are medicinally used for the treatment for antidiabetic, antibacterial, antistress, anti-inflammatory, ophthalmopathy, natural preservative, anticancer, antitussive, antiepileptic, antipyretic, hepatoprotective, nephroprotective, and anti-anaemic, immune modulatory.

Citrus is one of the most important commercial fruit crops in the world, and fruit weight, size, acidity and maturity index, harvest time, chemical and nutritional composition are important quality traits for fresh citrus consumption and acceptance by the citrus industry. An increase in the consumption of fruits and vegetables is associated with a decrease in the incidence of cardiovascular disease and reduce risks of certain cancers. Thus, citrus fruits have received much attention because of its nutritional and antioxidant properties and nowadays prevention of health problems through nutrition is promoted intensively, due mainly to the contribution of antioxidant compounds including vitamin C, phenolics compounds and carotenoids. Chemical variability of bioactive compounds and its relationship with genetic and climatic factors has been studied by diverse authors, and its contribution to the plant taxonomy has been reported. Organic acids, sugars and phenolic compounds are among the major compounds of citrus fruit pulp. Their nature and concentration largely affect taste characteristics and organoleptic quality. Organic acids and sugars vary according to species, varieties, and also environmental and horticultural conditions such as climate, rootstock, and irrigation. Also, the effect of citrus rootstocks on fruit nutritional quality has been studied by diverse authors. According to the kind of rootstock used, different morphological and

biological characteristics are obtained, including plant growth and fruit production, tree size, adaptation to certain soil conditions, size, texture, internal quality and maturity.

The content of vitamin C and other organic acids in fruits and vegetables can be influenced by various factors such as genotypic differences, preharvest climatic conditions and cultural practices, maturity and harvesting methods. Organic acids are a useful index of authenticity in fruit products since they have lower susceptibility to change during processing and storage than other components of fruits. At the same time, some organic acids may be used as indicators of ripeness, bacterial activity and adulteration. Previous reports of biochemical compounds have focused mainly on commercial varieties, and information regarding changes in biochemical constitutes of citrus fruit during ripening can be found in various reports. However, there is no comprehensive information regarding the changes in chemical bioconstituents during citrus fruits ripening in the same conditions of climate and field. We had studied the chemical variability of bioactive compounds in citrus pulp and juice and its relationship with genetic and climatic factors, and recently, we have evaluated the rind content of bioactive constituents (flavonoids, carotenoids, vitamin-C, essential oils and mineral composition) in several mandarin and orange cultivars from Mediterranean area.

MATERIALS AND METHODS:

Instruments used:-

Instrument	Specifications
HPLC	Waters, 2695 separation module
Software	Empower, Version 2.0
Detector	UV Visible detector
Analytical balance	Sartorius
UV-Visible spectrophotometer	Shimadzu (UV-2450)
Sonicator	Bioethics
PH meter	Cyber scan

Chemicals and Reagents:-

ortho Phosphoric acid (HPLC grade), Triethylamine (HPLC grade), HPLC grade water was used in buffer preperations.

Drug samples:-

Phyllanthus emblica , *Aleuria aurantica* and *Citrus limon* crude drugs were extracted from plants. Ascorbic acid (API)sample.

Collection of samples:-

Collection of peels and pulp and washed thoroughly and the collected sample undergone shade dried for 2-3 weeks. The collected peels were powdered after drying and the powdered sample were extracted with ethanol by soxhalation method.

Preparation of Buffer:

Mix 1ml TEA in 1litre water adjusts pH-2.5 with OPA.

Preparation of Mobile Phase:

Mix Methanol and Buffer in the ratio of 20+80. Filter through 0.45µ membrane filter paper.

Chromatographic condition:

Use suitable High Performance Liquid Chromatograph equipped with UV-visible detector.

Column : Luna C18, 250 mm x 4.6 mm, 5µm.

Wavelength : 243 nm

Injection Volume : 20µL

Column Temperature : Ambient

Flow rate : 1.0 ml/min

Retention time of Vit-C is about 2.9 min.

Preparation of Diluent:

Use Mobile Phase as a diluent.

Preparation of Standard Stock solution: Vit- C**:**

Weigh accurately about 5 mg of Vit- C working standard into a 100 mL volumetric Fenofibrate flask. Add 80 mL of diluent, sonicate to dissolve and dilute to volume with diluent.

Preparation of standard solution:

Further, dilute 5mL of standard stock solution to 50 mL with the diluent.

Preparation of sample solution:-

***PHYLLANTHUS EMBLICA*:**

Weighed accurately about 6.83mg of sample into a 10ml of volumetric flask, added 7ml of diluent, sonicated to dissolve and diluted to volume with diluent. Filtered through 0.45 nylon syringe filter. Further diluted 1.2ml of the above solution to 10ml with diluent**.**

***CITRUS AURANTIUM*:-**

Weighed accurately about 5.38mg of sample into a 10ml of volumetric flask, added 7ml of diluent, sonicated to dissolve and diluted to volume with diluent. Filtered through 0.45 nylon syringe filter. Further diluted 1.0ml of the above solution to 10ml with diluent.

***CITRUS LIMON*:-**

Weighed accurately about 4.44mg of sample into a 10ml of volumetric flask, added 7ml of diluent, sonicated to dissolve and diluted to volume with diluent. Filtered through 0.45 nylon syringe filter. Further diluted 1.0ml of the above solution to 10ml with diluent.

ASSAY:- The amount present was calculated by using following formula:-

$$A1/A2=W1/W2$$

A1= Area under the curve of sample 1

A2= Area under the curve of sample2

W1= conc. of known sample

W2= conc. of unknown sample

S. No.	Plant sample	Total amount of sample (mg)	Amount present (ppm)	% assay
1	*Phyllanthus emblica*	6.83	4.85	100.6
2	*Citrus aurantium*	5.38	5.0	99.6
3	*Citrus limon*	44.4	2.01	100.6

Method validation:-

Analytical method validation is a process of performing several tests designed to verify that an analytical test method is suitable for its intended purpose and is capable of providing useful and valid analytical data. There are several parameters that are considered in the method validation process as per International Conference of Harmonization (ICH) guidelines and as follow:

Linearity

Accuracy

Precision

1) Linearity:-

Linearity of detector response for *Phyllanthus emblica*, *Aleuria aurantica* and *Citrus limon* established for 6 concentrations ranging from 0 to 0.87µg/ml respectively. The concentration ranges of all those drugs in µg/ml were calculated. The slope, y-intercept, correlation coefficient (R^2) were calculated. The linearity data was given in table.

2) Accuracy:-

A study of accuracy was conducted by means of recovery studies. Recovery studies were carried out at 3 different levels. The preanalysed sample was spicked with 50%, 100% and 150% of mixed standard solution. The mixtures was analysed by the proposed method. The

study was carried out in triplicate. The average % recoveries of both the drugs were calculated and the results and chromatograms were shown below in table.

3) Intraday and interday precision:-

For injection repeatability, six injections from the same standard preparations were made and the relative standard deviation for the replicate injections was calculated. The readings of system precision were given in table no. For intraday precision, six individual preparations of *Phyllanthus emblica*, *Aleuria aurantica* and *Citrus limon* were prepared the relative standard deviation for the replicate injections was calculated. The readings of method precision were given in table.

4) Limit of detection

Limit of detection can be calculated using the following equation according to ICH guidelines:

$$\mathbf{LOD = 3\sigma/S}$$

Where σ= The standard deviation of peak areas of the drug and *S* is the slope of the corresponding calibration curve. The results are shown in table.

5) Limit of quantification

Limit of quantification can be calculated using the following equation according to ICH guidelines:

$$\mathbf{LOQ = 10 \times \sigma / S}$$

Where σ= The standard deviation of peak areas of the drug and *S* is the slope of the corresponding calibration curve. The parameters LOD and LOQ were determined on the basis of response and slope of the regression equation.

Isolation method of ascorbic acid in crude extract:-

- 2.5g of sample extract is decitrated using acetone.
- This solution is evaporated to dryness by N-propyl alcohol.

- To this was added an equal volume of petroleum ether and centrifuged, and the clear liquid divided into two parts.
- Lead acetate and methyl alcohol was added to one part.
- Yellow semi crystallized precipitate is produced.
- The yellow semi crystalline solid was centrifuged and dissolved in alcoholic HCl to remove lead. After evaporating the HCl, and sample stored in ice box.
- Second precipitate was treated in the same manner as the first.
- Remainder of clear liquid is precipitated by petroleum ether and propyl alcohol.
- This solution is evaporated to dryness by anhydrous ethyl acetate by overnight in ice box.
- The ethyl acetate extract was evaporated to equal volume of petroleum ether added.
- Light yellow semicrystalline material was produced and standard at room temperature.
- Needle like crystals (ascorbic acid crystals) were produced.

Identification of ascorbic acid crystals:-

- Identified by melting point determination of isolated crystals (190^0c).
- Identified by chemical test:-The test solution is treated with 0.2 ml of dinitrophenyl hydrazine then the solution is dissolved in sulphuric acid then yellow colour precipitate was formed.

Identified by FTIR spectroscopy:-

The ascorbic acid crystals of the three samples were subjected to FTIR. The FTIR interpretation results of the 3 samples were compared to the IR values of the standard ascorbic acid values. By observed that the interpretation values of the obtained yellow colored crystals were identified as ascorbic acid and it's having antioxidant activity.

RESULTS AND DISCUSSION:

Table 1:-calibration data for analysis of *Phyllanthus emblica*.

S. No	Concentration µg/ml	Peak area
1	0	0
2	0.75	74514
3	1.88	166194
4	3.75	317939
5	7.50	626870
6	9.38	769508
7	11.25	944076

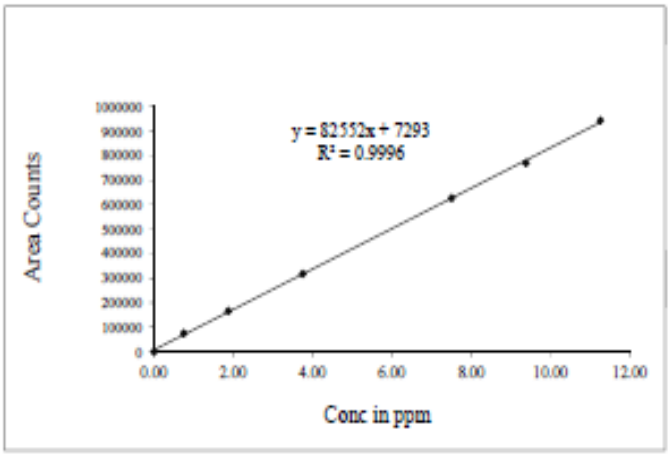


Table 2:-Calibration data for analysis of *Citrus limon.*

S. No	Concentrationµ/ml	Peak area
1	0	0
2	0.12	66261
3	0.31	162828
4	0.62	324421
5	1.25	635963
6	1.56	778519
7	1.85	951494

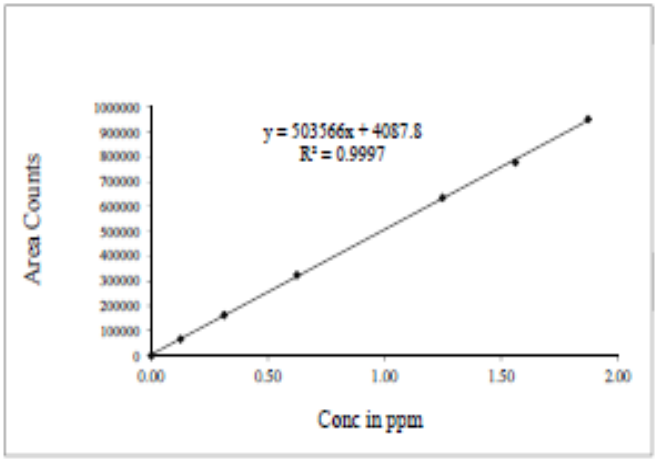


Table 3:-calibration data for *Citrus aurantia.*

S. No	Concentrationμ/ml	Peak area
1	0	0
2	0.12	28801
3	0.31	81743
4	0.62	132657
5	1.25	263144
6	1.56	323621
7	1.85	393941

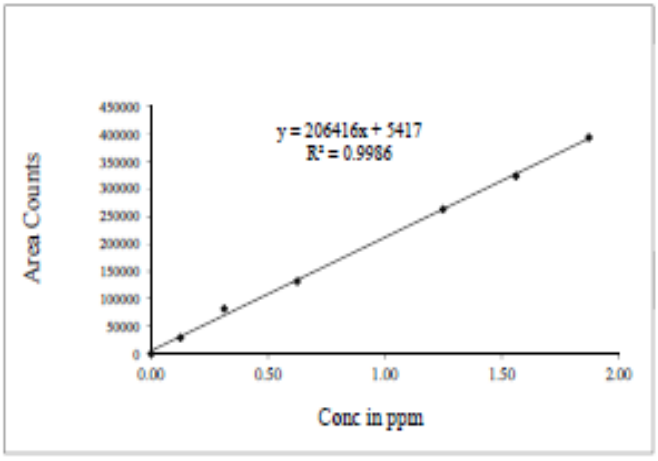


Table 4:- Accuracy results for *Phyllanthus emblica.*

Table 4). Data obtained after addition of known amount of pure drug to the sample.

S.No	Accuracy	Conc Ppm	Area	Amount Added	Amount Re covered	%Recovery	Results
1	50	50	312960	0.01997	0.02	100.2	Mean100.1
	50	50	318262	0.01999	0.02	100.1	SD 0.08
	50	50	317939	0.01996	0.02	100.2	%RSD 0.080
2	100	100	626870	0.04993	0.05	100.1	mean100.1
	100	100	625105	0.04992	0.05	100.1	SD 0.05
	100	100	626353	0.06994	0.05	100.2	%RSD 0.050
3	150	150	926972	0.06994	0.07	100.1	Mean100.1
	150	150	929190	0.06998	0.07	100.0	SD 0.04
	150	150	929425	0.06993	0.07	100.1	%RSD0.040

Table 5:- Accuracy results for *Citrus limon*.

Table 5). Data obtained after addition of known amount of pure drug to the sample.

S. No	Accuracy	Conc. ppm	Area	Amount added	Amount re covered	% Recovery	Results
1	50	50	324723	0.00995	0.01	100.5	mean100.4
	50	50	324975	0.01996	0.01	100.4	SD0.10
	50	50	322278	0.01997	0.01	100.3	% RSD0.100
2	100	100	606755	0.01991	0.02	100.5	mean100.3
	100	100	609213	0.01992	0.02	100.4	SD 0.16
	100	100	605267	0.01997	0.02	100.2	%RSD 0.160
3	150	150	948307	0.0 3997	0.04	100.1	Mean100.1
	150	150	944071	0.03994	0.04	100.2	SD 0.05
	150	150	942617	0.02998	0.03	100.3	%RSD0.050

Table 6:- Accuracy results for *Citrous aurantium*.

Table 6). Data obtained after addition of known amount of pure drug to the sample.

S. No	Accuracy	Conc. ppm	Area	Amount added	Amount re covered	% Recovery	Results
1	50	50	131829	0.01	0.01	100.0	mean:100.3
	50	50	131089	0.0099	0.01	100.2	SD 0.31
	50	50	131657	0.0099	0.01	100.6	%RSD 0.310
2	100	100	262810	0.0199	0.02	100.5	mean100.7
	100	100	265584	0.0199	0.02	100.6	SD 0.29
	100	100	265186	0.0198	0.02	100.5	%RSD 0.290
3	150	150	399621	0.0 298	0.03	100.7	Mean100.6
	150	150	399244	0.0299	0.03	100.3	SD 0.19
	150	150	393644	0.0298	0.03	100.7	%RSD0.190

Precision:-

System precision data

Table:-7 System precision data for *Phyllanthus emblica*.

S. No	RT	Area
1	3.192	646591
2	3.185	651945
3	3.183	654120
4	3.185	648594
5	3.183	661192
6	3.180	658366

Mean:-653468

% RSD:-0.859

Table:-8 System precision data for *Citrus limon*.

S. No	RT	Area
1	3.244	646591
2	3.242	651945
3	3.240	654120
4	3.244	648594
5	3.242	661192
6	3.240	658366
Mean:-653468		
%RSD:-0.859		

Table:-9 System precision data for *Citrus aurantium*.

S. No	RT	Area
1	3.244	646591
2	3.242	651945
3	3.240	654120
4	3.244	648594
5	3.242	661192
6	3.240	658366

Mean: 653468

%RSD: 0.859

Method precision data:-

Table:-10 Method precision data for Citrus limon.

S. No	RT	Area
1	3.247	604302
2	3.229	606857
3	3.223	605267
4	3.244	602497
5	3.223	609136
6	3.233	609236
Mean:-605673		
%RSD:-0.35		

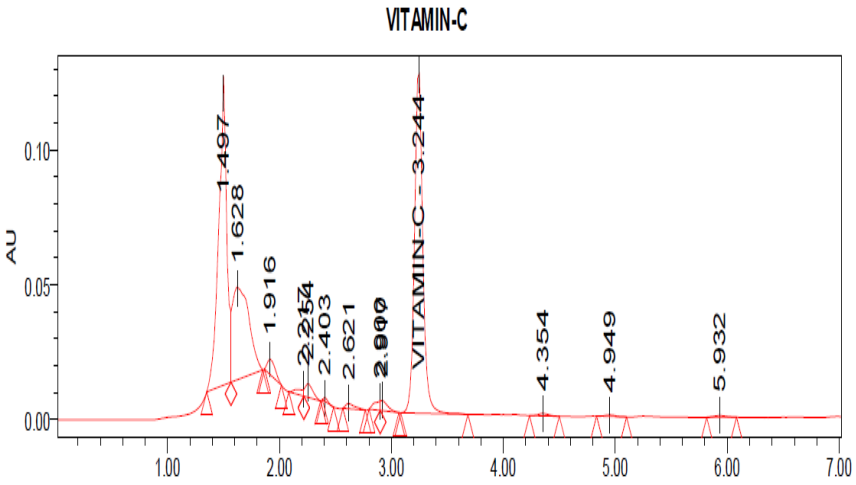
Table:-11 Method precision data for *Phyllanthus emblica*.

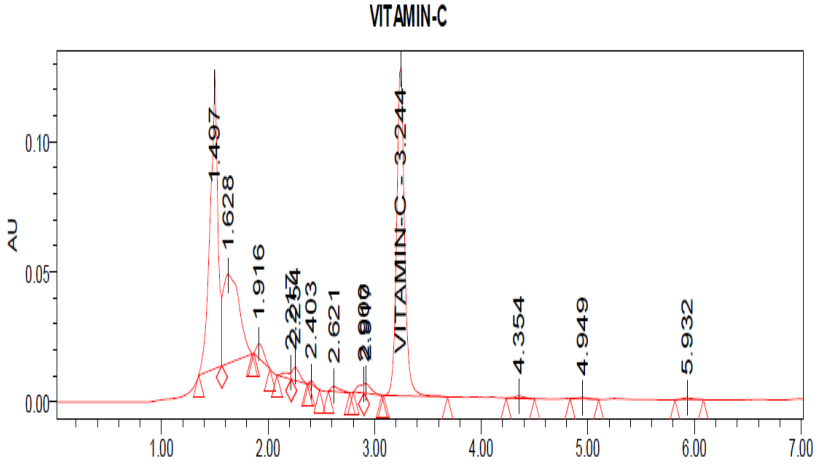
S. No	RT	Area
1	3.191	613131
2	3.181	617707
3	3.186	615930
4	3.192	618319
5	3.186	615171
6	3.185	614144
Mean:-616924		
%RSD:-0.29		

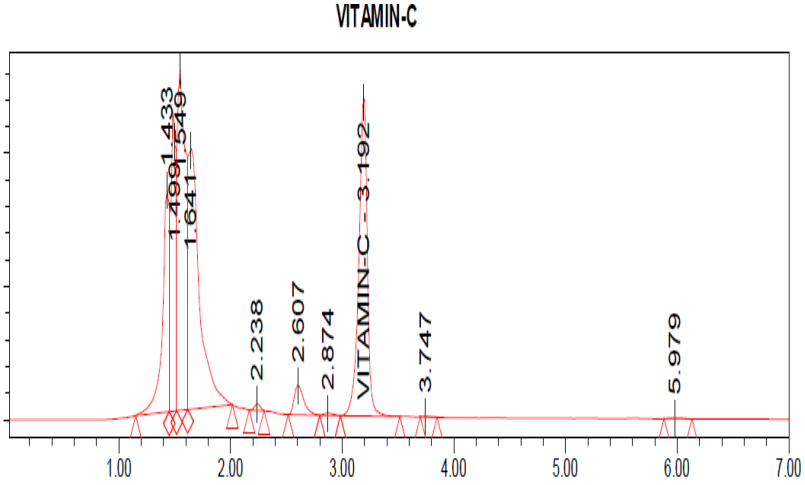
Table:-12 Method precision data for *Citrus aurantium*.

S. No	RT	Area
1	3.20	262810
2	3.189	265589
3	3.199	265186
4	3.186	263149
5	3.207	260897
6	3.186	260941
Mean:-266543		
%RSD:-0.74		

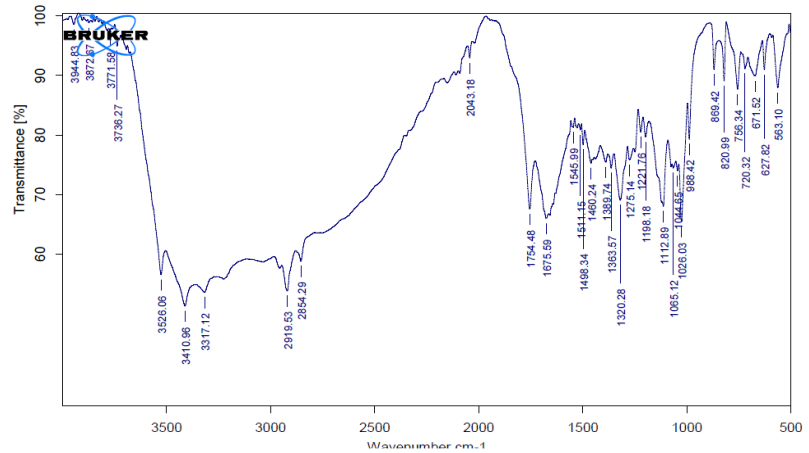
Graph for assay data for *Phyllanthus emblica, Citrus limon and Citrus aurantium*.

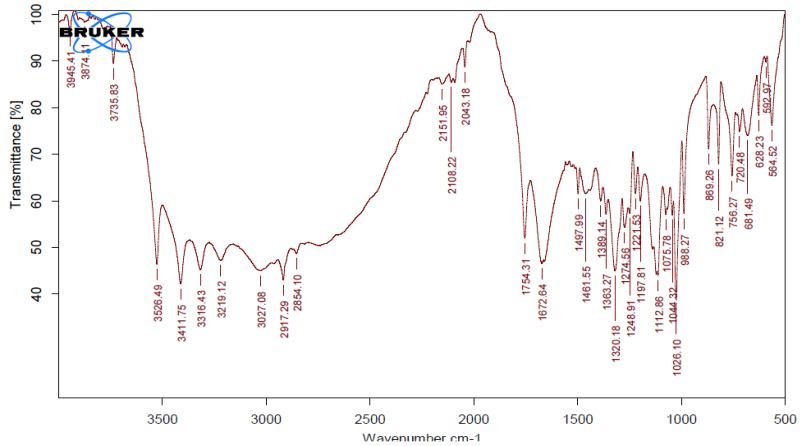






FTIR Graphs for isolated samples of *Phyllanthus emblica, Citrus limon and Citrus aurantium.*





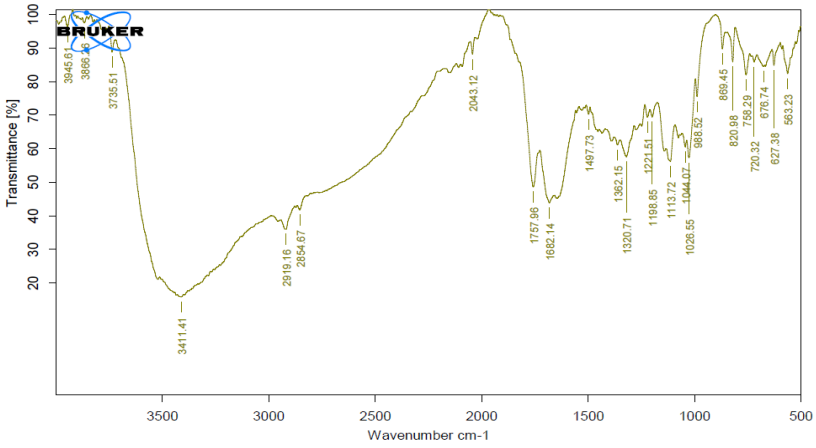


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Table-13: Interpretation values.

Functional group	Std Vitamin C	*Phyllanthus emblica*	*Citrus Aurantium*	*Citrus limon*
C-H str aromatic	3027.08	2919.53	2919.11	2919.16
C=O str ketone	1672.64	1754.48	1698.18	1682.14
C=O str acid	1461.55	1498.34	1496.13	1497.73
O-H str acid	1754	1754.48	1773	1757.96
O-H phenol	2917.29	2854.14	2881.25	2919.16
O-H str alcohol	3526.49	3526.06	3662.94	3411.41

Table-14: Validation parameters of *Phyllanthus emblica* by HPLC.

PARAMETER	ACCEPTANCE CRITERIA	*PHYLLANTHUS EMBLICA*
Linearity Range Correlation Coefficient	Correlation coefficient $r^2 > 0.999$ or	$r^2 = 0.999$
System Precision	RSD < 2%	%RSD = 0.859
Method precision	RSD < 2%	%RSD = 0.29
Accuracy	Recovery 98- 102% (individual)	% recovery=100.1

Table-15: Validation parameters of *Citrus aurantium* by HPLC.

PARAMETER	ACCEPTANCE CRITERIA	*CITRUS AURANTIUM*
Linearity Range Correlation Coefficient	Correlation coefficient $r^2 > 0.999$ or	$r^2 = 0.999$
System Precision	RSD < 2%	%RSD = 0.859
Method precision	RSD < 2%	%RSD = 0.74
Accuracy	Recovery 98- 102% (individual)	% recovery=100.5

Table -16: Validation parameters of *Citrus limon* by HPLC.

PARAMETER	ACCEPTANCE CRITERIA	*CITRUS AURANTIUM*
Linearity Range Correlation Coefficient	Correlation coefficient $r^2 > 0.999$ or	$r^2 = 0.999$
System Precision	RSD < 2%	%RSD = 0.859
Method precision	RSD < 2%	%RSD = 0.35
Accuracy	Recovery 98- 102% (individual)	% recovery=100.3

Assay parameters of *Phyllanthus emblica, Citrus limon and Citrus aurantium.*

NAMES	CONCENTRATIONS (mg/ml)	AREA	PERCENTAGE %
Vitamin c	5	646591	100
Phyllanthus emblica	6.83	626353	100.6
Citrus limon	4.44	645961	99.6
Citrus aurantium	53.8	261023	100.6

CONCLUSION:

A high – performance liquid chromatography (RP_HPLC) for the identification of Flavonoid (ascorbic acid) in *Phyllanthus emblica, Citrus aurantium* and *Citrus limon* was developed and validated for the estimation of vitamin C in *Phyllanthus emblica, Citrus aurantium* and *Citrus limon* in crude extract form. All the results were within the limits. The developed methods were validated as per ICH guidelines.

Good agreement was seen in the assay results of crude extract by developed methods. Hence it can be concluded that the proposed methods were good approach for obtaining reliable results. The developed method can be used for differentiation and quality evaluation of ascorbic acid from different sources. Synthetic antioxidants have side effects such as liver damage and carcinogenesis. Therefore, there is a need for isolation and characterization of natural antioxidant having less or no side effects, for use in foods or medicinal materials in order to replace synthetic antioxidants.

REFERENCES:

1. Olufunmilayo Sade Omoba 1,*, Rebeccah Olajumoke Obafaye 1,†*Antioxidants* 2015, *4*, 498-512 HPLC-DAD Phenolic Characterization and Antioxidant Activities of Ripe and Unripe Sweet Orange Peels.
2. Shrikant R. Kulkarni Journal of Engineering Research and Studies E-ISSN 0976-791 research article analytical method development for chiral separations of fruity flavonoids using RP-HPLC with improved selectivity and sensitivity.
3. Mannan Hajimahmoodi1*, Ghazaleh Moghadda Tropical Journal of Pharmaceutical Research June 2014; 13 (6): 951-95 Bioactive compounds and antioxidant activity of *Rosa canina L.* biotypes from spontaneous flora of Transylvania.
4. Ioana Roman chemistry central journal 2013 research article.
5. Iness jabri karoui and brahim marzouik 2013; 345415 Bio med research international.
6. Antioxidant Activities of Orange Peel Extracts *A.E. Hegazy and M.I. Ibrahium* World Applied Sciences Journal 18 (5): 684-688, 2012.
7. Sawant L, Prabhakar B, Pandita N (2010) Quantitative HPLC Analysis of Ascorbic Acid and Gallic Acid in *Phyllanthus Emblica*. J Anal Bioanal Tech 1:111. doi: 10.4172/2155-9872.1000111.
8. Vitamin C content and antioxidant activity of the fruit and of the Ayurvedic preparation of *Emblica officinalis* Gaertn.P. Scartezzini a,⁎, F. Antognoni a, M.A. Raggi b, F. Poli a, C. Sabbioni Journal of Ethnopharmacology 104 (2006) 113–118.

9. Vilbett Briones-Labarca1,2*, Claudia Giovagnoli-Vicuña1Extraction of □-Carotene, Vitamin C and Antioxidant Compounds from *Physalis peruviana* (Cape Gooseberry) Assisted by High Hydrostatic Pressure *Food and Nutrition Sciences*, 2013, 4, 109-118.
10. *Munish Puri", Madan Lal Verma and Kiran Mahale* Processing of Citrus Peel for the Extraction of Flavonoids for Biotechnological Applications.