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Functional group analysis of lyophilized pulp and seed powder of Phalsa (*Grewia asiatica*)

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Abstract
Phalsa (*Grewia asiatica*) is an indigenous, underutilized, edible fruit of the family *Tiliaceae* which possess huge nutritional potential and great medicinal value. In the present study, an attempt has been made to establish the preliminary FT-IR profile of pulp and seed powder of phalsa fruit subjected to drying in lyophilizer. Lyophilized fruit powder (seed & pulp) were analysed in mid infrared region of FT-IR spectroscopy for functional group identification. Resulted IR spectrum revealed the presence of various functional groups *viz*: alcohol, alkanes, alkenes, alkyne, aldehyde, ketone, primary amine, carboxyl group, alkyl halide, halogen, bioactive fraction and aromatic rings in both the samples. Functional groups commonly present in the pulp and seed of fruit indicates similar uses of both portions in food and medicinal applications. This investigation will provide a basic database on preliminary information of chemical constituents and their scope for further study.

Keywords: Phalsa, pulp powder, seed powder, Lyophilizer, FT-IR, functional groups

Introduction
Functional group identification is prerequisite in phytochemical studies and it is a key step in the process of determining the chemical constituents of any material. Recently, Fourier Transform Infrared Spectroscopy (FT-IR) has become a simplest and most reliable analytical technique to determine the functional groups of unknown composition. Moreover, it is an established time saving, non-destructive, fast and sensitive method to characterize and identify functional groups (Kumar *et al*., 2014) [12]. It measures the vibrations of bonds within chemical functional groups and generates a spectrum that can be regarded as metabolic finger print of the sample (Dalavi and Patil, 2016) [6]. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum. By interpreting the infrared absorption spectrum obtained, the chemical bonds in a molecule can be determined (George and Shanmugam, 2014) [7].
Phalsa (*Grewia asiatica*) which is also known as star apple (Tiwari *et al*., 2014) [28] is a warm climate, underutilized minor fruit crop of North India. It belongs to the family *Tiliaceae* and most commonly cultivated in the states of Punjab, Haryana, Rajasthan, Uttar Pradesh and Madhya Pradesh (Singh *et al*., 2015) [24]. Phalsa are berry like small fruits with deep reddish brown or purplish red color containing one or more seeds (Rathore *et al*., 2008) [22]. Ripe fruits are astringent and stomachic (Ahaskar and Sisodia, 2006a) [1] in nature, exhibits excellent medicinal, nutritious, sensory and bio-functional properties owing to the presence of vitamin C and A in significant amount and other constituents like minerals, carotenes, dietary fibers, anthocyanin type cyanidin 3- glucoside, phytochemicals etc (Ahaskar *et al*., 2006b) [2]. Moreover, fruits also exert cooling effect in hot summers and also beneficial for treating heart disorders, diarrhoea and fever but the shelf life of fruit is very short, therefore it is considered suitable only for local marketing (Haq *et al*., 2012) [10]. Dried powder of phalsa possess nutritional value (carbohydrates, protein, fat, fiber, minerals and energy) four times as compared to fresh fruit and it is not toxic, so can be used for edible purpose (Rahman *et al*., 2013) [21]. Thus, dehydration forms of this fruit may be efficient alternatives for storage.
Phalsa seeds are edible and usually eaten along with the fruit as they are very delicate and tightly embedded inside the flesh of the fruit. Seeds are good source of minerals like potassium, calcium, phosphorus, copper, zinc, magnesium and unsaturated fatty acids like α-tocopherol & β-sitosterol (Haq *et al*., 2015) [9] and these can also be effectively used for the remediation of Pb (II) contaminated water (Siyal *et al*., 2012) [25]. Hidden potential of the fruit can be explored by drying the fruit through non- thermal technology (lyophilization) and by critically analyzing the various functional groups.
The popularity of freeze-drying over conventional drying and others is based on some well-known advantages like retention of morphological characteristics, heat sensitive

phytochemicals, high recovery of volatiles, high yield, longer shelf life, crispy products and reduced weight in storage during shipping and handling (Ciurzynska and Lenart, 2011) [11] which suggests freeze drying as a technique for dehydration of phalsa fruit for the preparation of good quality dry powder. Despite its so much medicinal and nutritional potential, very limited work is reported in literature on this fruit. Literature survey has revealed that no research has been done on functional group analysis of lyophilized pulp and seed powder of phalsa using FT-IR. Therefore present study is an attempt to assess the functional group of phytoactive compounds present in these samples which will provide the needed preliminary information about various functional and biochemical activities of this underutilized fruit.

Materials and Methods

Present work was carried out in the department of Food Technology, Guru Jambheshwar University of Science and Technology, Hisar (Haryana), India. Fresh mature purplish red fruits of Sharbati variety were procured from Central fruit farm, Hisar in the month of June and July 2016-17. Fruits were sorted on the basis of visual quality parameters just after arrival in Fruits and Vegetables Processing laboratory. Decayed and discolored fruits were discarded and selected fruits were thoroughly washed with running tap water to remove adhering dust particles. After washing, fruits were kept in open trays and subjected to removal of water droplets from outer surface followed by storing in refrigerator (4 ± 1 °C) for few hours. Just before further processing, the fruits were removed from the refrigerator and kept at ambient temperature (30 - 35 ^{0}C) till it reaches its normal state. Then fruit flesh and seed were manually separated with the help of stainless steel knife. Further, 500 g fruit pulp and 200 g seeds were uniformly spread in different petriplates and kept for freeze drying at -70 °C until constant weight was achieved (24 hours). Fine powder of both (pulp and seed) was prepared separately using commercial grinder and was stored in air tight glass bottles at refrigeration temperature (4 ± 2 ^{0}C) for further analysis. All the chemicals used for this particular study were of analytical grade.

FT-IR Spectroscopic Analysis

Functional groups were characterized using Fourier Transform spectrophotometer (Perkin Elmer (Spectrum BX), following the method of Mohan (2005) [16]. Prior to analysis the powdered samples were stored in desiccators to avoid shifts in the spectra due to interference by moisture gain. FT-IR spectra were recorded at room temperature in the frequency region 4,000 to 400 cm^{-1} (mid infrared region) at the resolution of 4 cm^{-1}. Samples were taken in triplicates and spectra readings for each sample were obtained separately. Powdered fruit pulp and seed (2 ± 0.2 mg) were properly mixed with 100 mg of potassium bromide (KBr, FT-IR grade) individually and mixture was transferred to a mould. The material was then compressed in hydraulic press in order to produce a clear transparent disc of 13 mm diameter and 1 mm thickness which were then removed and immediately placed between interferometer and detector in the sample holder of the spectroscope. Infrared spectrum was Fourier transformed and recorded in the transmission mode. Figure 1 and 2 shows the Interferogram obtained from FT-IR spectroscopy between wave number (400 - 4000 cm^{-1}) and absorption. All the spectral values were expressed in percent transmittance (Ciurzynska and Lenart, 2011) [11]. The spectral data obtained were compared with the reference chart to identify the functional groups present in the samples.

Results and Discussion

In the presented work, FT-IR spectrum was used to identify the functional groups of the active components in phalsa pulp powder and seed powder, based on the peak values in the region of infrared radiation by comparing the obtained spectra with that reported by (Stuart, 2004) [26]. The resultant spectral peaks were analyzed for the occurrence of characteristic functional groups that might represent the chemistry of various compounds present in the fruit pulp. FT-IR peak values and corresponding functional groups of both samples are depicted in Tables 1 and 2 and the FT-IR spectra profiles were shown in Fig. 1 and 2. The FT-IR spectrum of pulp and seed powder each consisted of 29 and 24 major peaks at the range of 3405.95 – 521.90 cm^{-1} and 3424.14 - 414.69 cm^{-1} respectively. Most of the functional groups observed as per their peaks are similar in both the samples with a little difference only in their wave numbers. The spectral analysis reveals that phalsa fruit and seed display several functional groups like alcohol, alkanes, aromatic compounds, carboxylic groups, alkenes, amines, amides, aldehydes, ketones and halogen compounds. Aromatic compounds, phenols, aldo-ketogroup peak indicate the presence of polyphenols of which flavonoids, coumarins, anthroquinones and phenolic compounds are commonly present in the plants attribute to their antioxidant power (Sunila *et al*., 2016) [27].

The bands in pulp and seed powder of phalsa (Fig. 1, 2) were observed at 3405.95 cm^{-1} and 3424.14 cm^{-1} which represented occurrence of amines and alcohol compounds. The band at 2926 cm^{-1} represented the cycloalkanes. Peaks obtained around 2363 and 2345 cm-^{1} are corresponding to nitriles. Peaks obtained at 1735 cm^{-1} and 1726 cm^{-1} indicated the alkenes. Peak observed at 1438 cm^{-1} showed the occurrence of alkanes (Table 1, 2). Remaining peaks observed at 868, 818, 778 and 595 cm^{-1} (Fig. 1, 2) indicated primary amines, aromatics or aliphatic amines and alkyl halides collectively.

Table 1: Functional Groups Identified By FT-IR Spectroscopy for Lyophilized Pulp Powder of Phalsa

S. No.	Peak value (cm^{-1})	Stretching	Functional group
1	3405.95	N-H stretching	Amines
2	2926.64	C-H stretching	Cycloalkanes
3	2363.73	C≡N or C≡O stretching	Nitriles or alkynes
4	2345.33	C≡O or C≡N stretching	Nitriles or alkynes
5	1970.80	C=O stretching	Carbonyl
6	1735.75	C=C stretching	Alkenes
7	1726.74	C=C stretching	Alkenes
8	1654.16	C=O stretching	Ketones
9	1629.38	COO^- antisymmetric stretching	Polygalacturonic acid, carboxylate (pectin ester group)
10	1618.87	COO^- antisymmetric stretching	Polygalacturonic acid, carboxylate (pectin ester group)
11	1518.00	C-N	Nitro compounds
12	1512.26	N-H stretching	Nitro compounds
13	1438.20	C-H bending	Alkanes
14	1420.39	C-H stretching	Alkanes
15	1408.16	C-N bend	Amides
16	1235.45	C-N stretching	Bioactive fraction
17	1101.40	C=O stretching	Ketones
18	1057.01	C-O-C stretching	Ethers or related compounds
19	919.79	C-O stretching	Alkyl halides
20	897.48	C1-H bending	Xyloglucan (polysaccharide)
21	868.23	C-N bend	Primary amines/ Carbon-Nitrogen Compounds
22	818.04	C-H bending	Aromatic or aliphatic amines
23	778.84	C- Cl stretch	Alkyl halides
24	701.74	C-Br stretching	Halogen compounds
25	630.23	C-H stretching	Alkynes
26	618.19	C-H stretching	Halogens (iodo compounds)
27	596.92	C-Br stretching	Alkyl halides (bromide, iodide, chloride)
28	562.68	C-Br stretching	Alkyl halides (bromide, iodide, chloride)
29	521.90	C-Br stretch	Alkyl halides (bromide, iodide, chloride)

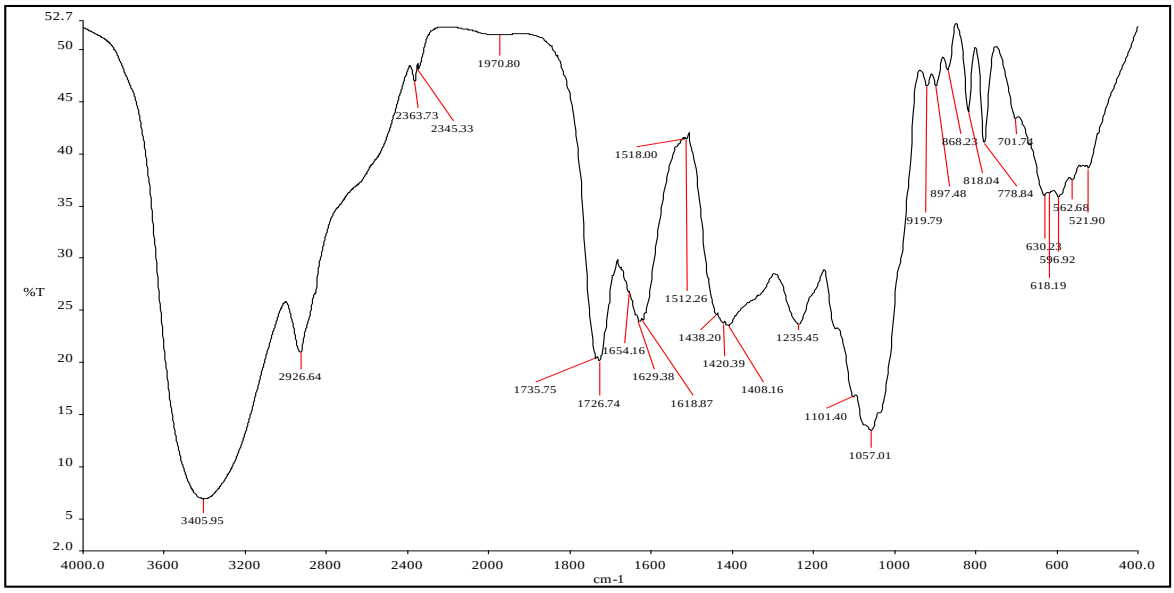

Fig 1: FT-IR chromatogram of pulp powder of phalsa

In case of pulp powder (Fig. 1), the peak observed at 1970.80 cm^{-1} showed carbonyl group. The band at 1654.16 cm^{-1} indicated the ketone group and 1629.38, 1618.87 cm^{-1} indicated pectin. Peaks at 1518.00 and 1512.26 cm^{-1} signified nitro compounds. Remaining peaks observed at 1420.39, 1408.16, 1235.45, 1101.40, 1057.01, 919.79, 897.48 and 630.23 cm^{-1} are corresponding to the alkene, amide, bioactive fraction, ketone, ether, alkyl halide, xyloglucan (polysaccharide) and alkyne respectively. Peaks observed at 701.74, 618.19, 562.68 and 521.90 cm^{-1} confirmed the presence of halogens as depicted in table 1.

In case of seed powder (Fig. 2) the peaks observed at 3011.35, 2855.60, 2132.01 and 1743.97 cm^{-1} confirmed the presence of aromatic compounds, carboxylic acid, nitrile and alkyl ester respectively. The peaks around 1719.95, 1647.22, 1499.38 and 1491.91 cm^{-1} denoted the ester, amino acid, aromatic compound and halogen group. Remaining peaks at 1459.49, 1243.18, 1156.82, 1053.85 and 414.69 cm^{-1} confirmed the alkane, pectin, amino acid, esters and chloride functional groups as shown in Table 2 (Stuart, 2004) [26]. Peaks obtained at 1518.00 cm^{-1} and 1512.26 cm^{-1} (Fig. 1) indicated about the β-carotene (Nikbakht, 2011) and the catechin respectively. FT-IR spectra obtained at 868.23 cm^{-1} (Fig. 1, 2) and at 1235.45 and 1618.87 cm^{-1} (Fig. 1) represents gallic acid (Gorinstein *et al*., 2011) [8].

Table 2: Functional Groups Identified By FT-IR Spectroscopy for Lyophilized Seed Powder of Phalsa

S. No.	Peak value (cm^{-1})	Stretching	Interpretation
1	3424.14	O-H or N-H stretching	Alcohol or amines
2	3011.35	N-H stretching	Aromatic compounds
3	2926.69	C-H stretching	Cycloalkanes
4	2855.60	C-H stretching	Carboxilic acids
5	2363.92	C≡N or C≡C stretching	Nitriles or alkynes
6	2345.49	C≡C or C≡N stretching	Nitriles or alkynes
7	2132.01	C≡C or C≡N stretching	Nitriles or alkynes
8	1743.97	C=O stretching	Alkyl esters
9	1736.25	C=C stretching	Alkenes
10	1726.03	C=C stretching	Alkenes
11	1719.95	C=O stretching	Esters
12	1647.22	C=O stretching	Amino acids
13	1499.38	C=C stretching	Aromatic compounds
14	1491.91	C-F stretching	Halogen
15	1459.49	C-H stretching	Alkanes
16	1438.13	C- H bending	Alkanes
17	1243.18	C-O stretching	Carbohydrates (pectins)
18	1156.82	N-H bending	Amino acids
19	1053.85	C-O-C stretching	Ethers or esters
20	868.65	C-N bending	Primary amines/ Carbon-Nitrogen Compounds
21	818.34	C-H stretching	Aromatics or aliphatic amines
22	778.05	C-Cl stretching	Alkyl halides
23	595.74	C-Br stretching	Halogens (Bromo compounds)
24	414.69	C-Cl stretching	Chlorides

It can be concluded from this study that this underutilized fruit (pulp and seed) is a valuable source of many compounds which can be isolated for further utilization. Every compound is commercially important and can be utilized for various products formation. For example: Aromatic amines are used in rubber, textile and dye industries. Industrially, amines are very important because of their use in removal of carbon dioxide and hydrogen sulphide from natural gas. Many amine-rich proteins are bound to DNA and some neurotransmitters like epinephrine, dopamine are also amines (Nelson and Cox, 2000) [17]. Amines and amides are the main groups of protein synthesis (Pillai and Nair, 2014) [19].

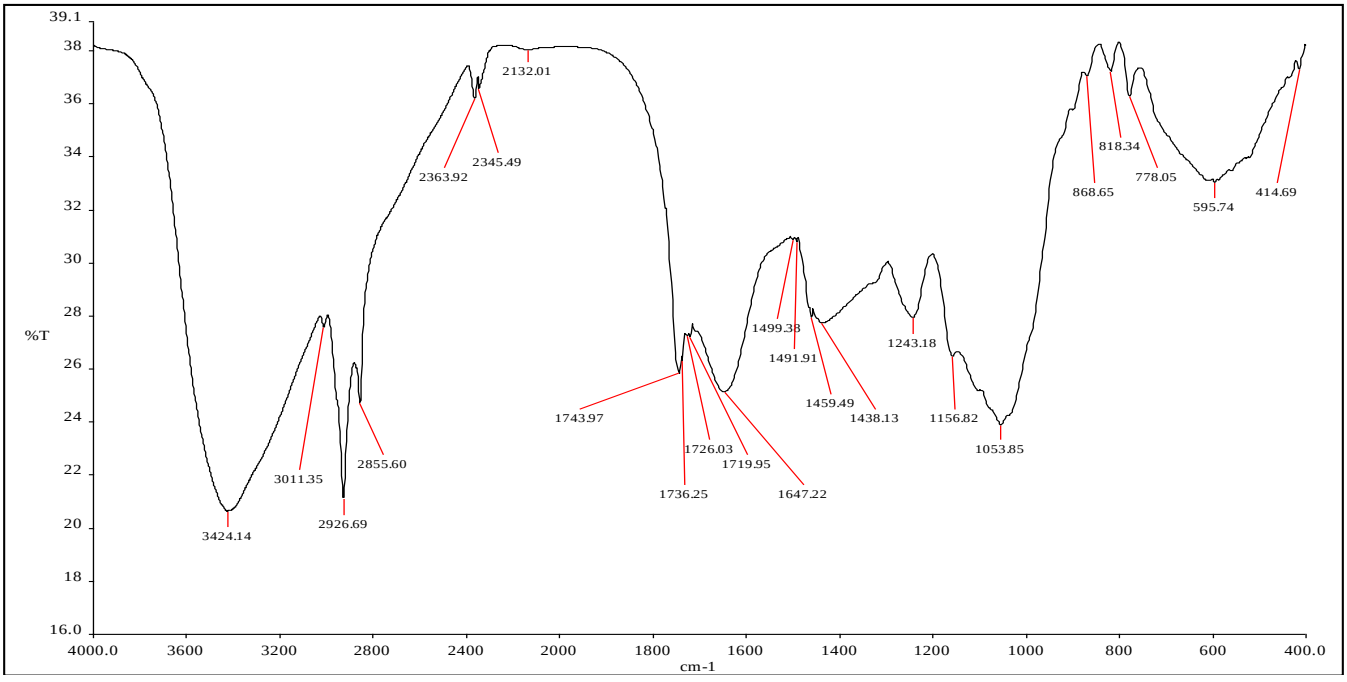

Fig 2: FT-IR chromatogram of seed powder of phalsa

Phenols are of great importance as they act as antioxidants to protect the human body from the oxidative stress, which is responsible for many diseases and also form the integral part of the cell wall structure (Robards *et al*., 1990) [23]. Antimicrobial, antiapoptotic, anthelmintic, antidiarrhoeal properties are also possessed by phenols. These are also found useful in dettol and cresol preparation (Cowan, 1999) [5]. Pectins are useful for the food industry, desserts, dairy products, pharmaceuticals and soft drinks (May, 1990) [14].

The alkanes are constituent of plant cuticle and epicuticular wax of many species. They possess protecting behavior in the plant against water loss, harmful insects and prevent the leaching of important minerals by rain (Baker, 1982) [3]. Alkenes serve as raw materials for the manufacture of alcohols and aldehydes and are of great importance in the

manufacture of plastics, as fuel and illuminant. These play important role in artificial ripening of fruits also (Pillai and Nair, 2014) [19]. Alkynes are highly bioactive as nematocides and exhibit antitumor, antiviral, antifungal properties. These are the important constituent of some pharmaceutical drug like contraceptive norethynodrel and some acids like tartaric acid (Walker *et al*., 1992) [29].

Carboxylic acids have biological importance. These help in the formation of fat in the body and act as strong antibacterial agents. These are also important constituent in pharmaceutical products in curing ulcers, jaundice, headache, fever, pain in liver, wound in cattle, treatment of edema and rheumatic joint pains. The non-aspirin pain reliever ibuprofen is also a carboxylic acid (Pillai and Nair, 2014) [19]. Aldehydes with combination of phenol produce resins (e.g., Bakellite) and esters in combination with volatile oils are responsible for the production of pleasant aroma of fruits (Janakiraman *et al*., 2011) [11]. Sulfur compounds are used in disinfectants and dental creams and are important constituent of amino acids of proteins, volatile oils and sulfates (Lowe, 1937) [13]. Halogen compounds yield chlorinated alkaloids via. Chlorinated tryptophan and Chlorates acts as disinfectants (Walker *et al*., 1992) [29]. Bromides help eosinophils (white blood cells) in the generation of anti-parasitic brominating compounds by the action of eosinophil peroxidase (Mayeno *et al*., 1989) [15].

There was no absorbance in between 2220-2260 cm^{-1} which indicates that no cyanide group is present in both the samples which mean powders are non toxic (Praveen and Nair, 2015) [20]. The results of the present study suggest that phalsa seed and fruit can be used for many functions in the near future. In addition, many functional groups can be identified by their characteristics vibration frequencies making the IR spectrum simplest and most reliable method of assigning a compound to its class (Sunila *et al*., 2016) [27].

Conclusion

FT-IR is a very quick, reliable and cheaper analytical technique which provides an excellent means to visualize the chemical compositions of lyophilized pulp and seed of phalsa fruit. The results of the present study provides an insight into the functional groups present in this fruit and results depicted that it is a rich source for many phyto-constituents which can be isolated, characterized and screened for various activities. These functional compounds can be utilized in many pharmaceutical, nutraceutical, functional food and cosmetics industries. The presence of various bioactive compounds in both pulp as well as seed justifies that this is a fruit of phyto-pharmaceutical importance and might be a potential source of natural antioxidants. However, further studies will need to be undertaken to find out the structural analysis of compounds by applying recent analytical tools so that hidden potential of this miracle fruit can be explored.

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References

1. Ahaskar M, Sisodia R. Modulation of radition induced biochemical changes in brain of swiss albino mice by *Grewia asiatica*. Asian J Exp Sci. 2006; 20(2):399-404.
2. Ahaskar M, Sharma KV, Singh S, Sisodia R, Radioprotective effect of fruit extract of *Grewia asiatica* in swiss albino mice against lethal dose of γ-irradiation. Asian J Exp. Sci. 2006; 21(2):295-308
3. Baker EA. Chemistry and Morphology of Plant Epicuticular Waxes, Academic Press London, 1982, 139-165
4. Ciurzyńska A, Lenart A. Freeze-drying – application in food processing and biotechnology – A review. Pol J Food Nutr Sci. 2011; 61(3):165-171
5. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999; 12(4):564-582
6. Dalavi C, Patil S, FTIR spectroscopic screening of phytochemicals of two medicinally important species of solanum used in the preparation of dashmula formulation. Int J Pharm Sci Rev Res. 2016; 36(2):112-120
7. George B, Shanmugam S. Phytochemical screening and antimicrobial activity of fruit extract of *Sapindus mukorossi*. Int J Curr Microbiol App Sci. 2014; 3(10):604-611
8. Gorinstein S, Poovarodom S, Leontowicz H, Leontowicz M, Namiesnik J, Vearasilp S *et al*. Antioxidant properties and bioactive constituents of some rare exotic Thai fruits and comparison with conventional fruits In vitro and in vivo studies. Food Res Int. 2011; 44:2222–2232.
9. Haq MZU, Ahmad S, Imran I, Sli SE, Moga M. Compositional study and antioxidant capacity of *Grewia asiatica L*. seeds grown in Pakistan. C R Acad Bulg Sci, 2015, 68(2).
10. Haq MZU, Shahid SA, Muhammed S, Qayum M, Khan I, Ahmad S. Antimalarial, antiemetic and antidiabetics potential of *Grewia asatica* L. leaves. J Med plants Res 2012; 6(16):3213-3216
11. Janakiraman N, Satish SS, Johnson M. UV-VIS, FT-IR spectroscopic studies on *Peristrophe bicalyculata* (Retz.) Nees. Asian J Pharm Clin Res. 2011; 4(4):125-129.
12. Kumar SNK, Suresh M, Kumar SA, Kalaiselvi P. Bioactive compounds, radical scavenging, antioxidant properties and FTIR spectroscopy study of *Morinda citrifolia* fruit extracts. Int J Curr Microbiol App Sci. 2014; 3(2):28-42.
13. Lowe B, Experimental Cookery from the Chemical and Physical Standpoint John, Wiley & Sons, 1937.
14. May CD. Industrial Pectins: Sources, production and applications. Carbohydr Polym. 1990; 12:79-99.
15. Mayeno AN, Curran AJ, Roberts RL, Foote CS. Eosinophils preferentially use bromide to generate halogenating agents. J boil Chem. 1989; 264(10):5660-5668
16. Mohan J. Organic Spectroscopy Principles and Applications, Narosa publishing House, Daryagani, Delhi, 2005.
17. Nelson DL, Cox MM, Lehninger, Principles of Biochemistry. Worth Publishing, New York, 2000.
18. Nikbakht AMT, Hashjin TR, Malekfar, Gobadian B. Nondestructive determination of tomato fruit quality parameters using Raman spectroscopy. J Agr Sci Tech 2011; 13:517-526.
19. Pillai LS, Nair BR. Functional group analysis of *Cleome viscosa* L. and *C. burmanni* W. & A. (Cleomaceae) extracts by FT-IR. J Pharmacogn Phytochem. 2014; 2(6):120-124.
20. Praveen RP, Nair AS. Functional group analysis for methanolic extracts of root, fruit and callus of *Myxopyrum smilacifolium* Blume Int J Pharm Sci Rev Res. 2015; 33(2):1-4.

21. Rahman AU, Fatima N, Imran H, Saleem N, Sohail T, Raif M. Nutritional and toxicological evaluation of *Grewia asiatica* (Phalsa) powder used as a summer drink. J Chem Soc Pakistan. 2013; 35(2):321-324
22. Rathore AC, Raizada, Jayaprakash J. Performance of phalsa (*Grewia Subinaequalis* Lin.) through integrated nutrient and canopy management on saline soils of the Indo-Gandetic plains. Indian J Soil Cons. 2008; 36(1):42-47.
23. Robards K, Prernzler PD, Tucker G, Swatsitang P, Glover W. Phenolic compounds and their role in oxidative processes in fruits. Food Chem. 1990; 66:401-36
24. Singh KK, Tomar YK, Rawat JMS, Study on the effect of different planting time and various concentration of IBA on the rooting of phalsa (*Grewia asiatica*) stem cutting under different growing condition. Int J curr Res. 2015; 7(2):12674-12679
25. Siyal AN, Memom SQ, Khaskheli MI. Optimization and equilibrium studies of Pb (II) removal by *Grewia asiatica* seed: a factorial design approach. Pol J Chem Technol. 2012; 14(1):71-77.
26. Stuart B. Infrared Spectroscopy: Fundamentals and Applications, John Wiley & Sons, 2004.
27. Sunila AV, Kumar AVS, Babu DKV, Murugan K. Comparison of FTIR fingerprints in the fruits of *Pouteria campechiana* (Kunth) Baehni at different developmental stages. Int J Pure App Biosci. 2016; 4(1):226-234.
28. Tiwari DK, Singh D, Barman K, Patel VB. Bioactive compounds and processed products of phalsa (*Grewia subinaequalis* L.) fruit. Popular Kheti. 2014; 2(4):128-132
29. Walker S, Landovitz R, Ding WD, Ellestad GA, Kahne D. Cleavage behavior of calicheamicin gamma[1] and calicheamicin. T. Proc Natl Acad Sci USA. 1992; 89(10):4608-12.