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Evaluation of saponins in *Aloe vera* by High-performance liquid chromatography and Fourier transform infrared spectroscopy

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Abstract
Aloe vera is a potent source of active ingredients. Saponins are considered the most active secondary metabolites, accountable for the pharmacological properties of *Aloe vera*. The conventional maceration method was optimized to prepare the saponin-rich extract. HPLC method was optimized with a detection wavelength of 235 nm, 1.0 ml/min flow rate with 35% acetonitrile as mobile phase, and injection volume of 10 µl at 20 °C column temperature. The method was calibrated by analyzing five standard samples with a known amount of purified saponin concentration 0.2 mg/ml to 1 mg/ml with good linear regression (r2=0.99) and a retention time at 1.9 min. The estimated saponin content ranged from 0.021 mg/g to 0.414 mg/g in different tissues of *Aloe vera*. Phytochemical screening of crude extracts was done by using Fourier Transform Infrared (FTIR) spectrometer. FTIR spectra of all the test samples of Aloe vera, i.e., whole leaf, gel, leaf peel, sap, and root, showed –OH, -C=O, C-H, and C=C absorptions, which are typical characteristics group of saponins. So tissue-specific quantification of saponin would be a quick reference for biochemical characterization in *Aloe vera*.

Keywords: Lentil, fusarium, fungicides, evaluation, neem

1. Introduction
Aloe vera (*Aloe barbendensis Miller*) is the most potent medicinal plant among more than 400 Aloe species (Chen *et al*., 2012) [1]. The plant contains a significant quantity of water and different bioactive ingredients, including anthraquinones, lignin, tannic acids, polysaccharides, glycoprotein, saponins, sterols, amino acids, salicylic acid, vitamins, enzymes, sugars, and various minerals (Femenia *et al*., 1999) [2]. These bioactive ingredients and the derived products have wide applications in many fields such as medicine, health care, cosmetics, and food (Chang *et al*., 2007; Wang, 2001) [3, 4]. Among these, saponins are a structurally diverse class of compounds occurring in many plant species, including aloe. Saponins are often subdivided into two main classes, the triterpenoids and the steroid saponins, both derived from the 30 carbon atoms containing precursor oxidosqualene by the action of various cyclases (Haralampidis *et al*., 2002; Kalinowska *et al*., 2005) [5, 6]. These are also used as viricidal, antibacterial, anti-inflammatory, and anti-leishmanial drugs (Lambert *et al*., 2011) [7]. For biochemical characterization and elucidation of bioactivities in *Aloe vera*, extensive research has been conducted predominantly using the techniques of spectroscopy and chromatography (Raksha *et al*., 2014) [8]. Chromatographic techniques have a more significant role in understanding natural product chemistry and discovering novel bioactive compounds of medicinal importance. High-performance liquid chromatography (HPLC) is the usual technique for determining individual components in Aloe vera leaf extracts. Reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detector was employed to analyze four saponins of *Achyranthes aspera* and *Cissus quadrangularis* (Talreja *et al*., 2017) [9]. Quantitative analysis by high-performance liquid chromatography (HPLC) was done to explore saikosaponin a and d in *Bupleurum falcatum* (Park *et al*., 2000) [10], novel saponins in Ilex paraguariensis (Gnoatto *et al*., 2005; Pavei, 2007) [11, 12], ginsenosides in *Panax notoginseng* (Liu *et al*., 2017) [13] and many more. There are also several examples of HPLC using reverse phase column for the analysis of aloin derivatives in *Aloe vera* (Okamura *et al*., 1996; ElSohly *et al*., 2007) [14, 15]. Fourier Transform Infrared (FTIR) spectroscopy is also proved as an essential technique for phytochemical analysis of saponins in medicinal plants, including *Albizia anthelmintica, Senna singueana, Maytenus senegalensis, Senna didymobotrya, Terminalia brownii, Prunus Africana.*

Entada leptostachya and *Rapanearho dodendroides* (Kareru *et al*., 2007) [16]. Isolation and characterization of saponins in Moringa oleifera, a plant having a wide range of medicinal uses with high nutritional value, was performed using HPLC and FTIR (Sharma and Paliwal, 2013) [17]. In the present research, the presence of saponins in different *Aloe vera* tissues is validated by High-performance liquid chromatography and Fourier Transform Infrared spectroscopy.

2. Material and Methods

2.1. Preparation of crude extract

High-performance liquid chromatography and Fourier Transform Infrared spectroscopy (FTIR) were performed to detect and quantify saponins in crude extracts of whole leaf, gel, sap, leaf peel, and root of *Aloe vera*. Extraction of crude saponins was carried out by adopting the conventional maceration method of Kwon *et al*. (2003) [18] with slight modifications. Whole leaf paste and gel were proceeded for lyophilization to dry and leaf peels, sap, and roots were dried at room temperature.The dried tissues were ground to form a powder and used for further methanol and butanol extraction of saponins. Five gram of powdered tissue of each sample type was taken and dissolved in 100 ml of 70% methanol and kept in a shaking incubator for 24 hours. The supernatant was filtered through Whatman filter paper (3.1).The residue was taken back and re-extracted one more time using fresh solvent, each time with the same conditions as above. The combined methanolic extracts (200 ml) were fractionated with butanol and water to get a saponin-rich fraction (Pasha *et al*., 2016) [19]. Dried butanol extract and standard saponin powder were further purified with diethyl ether. Stock solutions of the analyte (5 mg/mL) were prepared by dissolving suitable amounts of each pure substance in MiliQ water and stored at −20 °C.

2.2. High-performance liquid chromatography (HPLC)

HPLC grade solvents were purchased from Merck, Darmstadt, Germany. Ultrapure water was supplied by a Milli-Q water purifier system from Millipore. Saponins, as a standard, was purchased from Sigma-Aldrich, St. Louis, USA. Total yield and crude saponin content were determined by a high-performance liquid chromatography-diode array detector (HPLC-DAD) from Agilent technology containing reverse-phase chromatographic C18 column. Triplicate runs were carried out for each partitioning experiment for accuracy. The method was calibrated by analyzing five standard samples with a known amount of purified saponin of concentration 0.2 mg/ml to 1 mg/ml. The mobile phase consisted of water (65%) and acetonitrile (35%). The detection wavelength was 235 nm, and the flow rate was 1.0 mL/min. Each injection volume was 10 µL. The column temperature was maintained at 20° C.

2.3. Fourier Transform Infrared spectroscopy (FTIR)

Crude butanol extracts of whole leaf, gel, sap, leaf peel, and root of *Aloe vera*, in powder form, were mixed with potassium bromide (KBr) powder and further compressed to form a thin pellet for infrared examination in FT-IR Spectrophotometer (Model: Perkin Elmer Spectrum, BX II). Infrared spectra were recorded in the range of 3500 – 600 cm -1, and spectra obtained were used for further analysis.

3. Results and Discussion

3.1. Primary detection of saponins

A dry foam test was performed to check the presence of saponins qualitatively at the primary level for each test sample (Aziz *et al*., 2019) [20]. About 1 gram extract in powder form from each sample was dissolved in 5 ml distilled water in a test tube and heated in an incubator, and the froth appeared to be mixed with few drops of olive oil and shaken vigorously. The formation of emulsion indicates the presence of saponins.

3.2. Characterization of saponins by HPLC

Quantification of saponin content of *Aloe vera* whole leaf, gel, leaf peel, sap, and root was done using HPLC. The conventional maceration method was optimized to prepare crude saponin extract (Pasha *et al*., 2016) [19]. The method was calibrated by analyzing five standard samples with a known amount of purified saponin of concentration 0.2 mg/ml to 1 mg/ml. The average peak area of saponin was reported and used for quantification of different *Aloe vera* tissues. A calibration curve was plotted by taking the peak area against the concentration of each analyte. The goodness of fit of the standard saponin showed good linear regression (r2=0.99) in the range of concentration. The concentration of saponin in *Aloe vera* whole leaf, gel, leaf peel, sap, and root was calculated in respect of peak area concerning standard saponin of known concentration. The estimated saponin content ranged from 0.021 mg/g to 0.414 mg/g in different tissue of *Aloe vera*. The maximum saponin content of 0.414 mg/g was observed in *Aloe vera* sap, followed by 0.101, 0.072, and 0.065 mg/g in the whole leaf, gel, and leaf peel, respectively. Roots of *Aloe vera* contain the lowest saponin concentration of 0.021 mg/g.

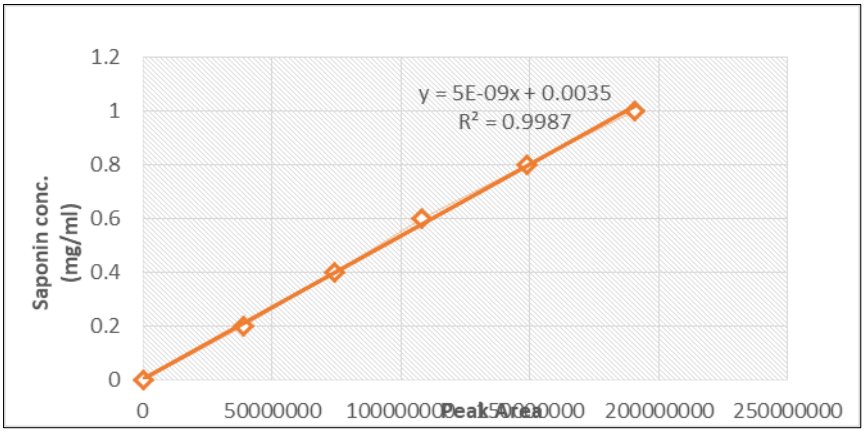

Fig 1: RP-HPLC calibration curve of different concentration of standard saponin

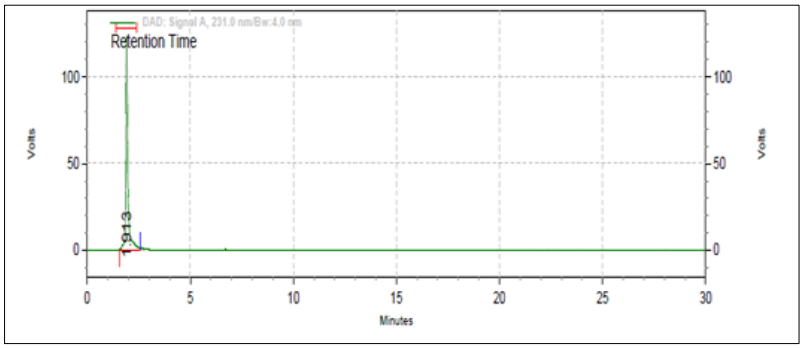

Fig 2: RepresentingC-18 RP-HPLC chromatograms of standard saponin, whole leaf, Gel, Peel, Sap, and Root respectively

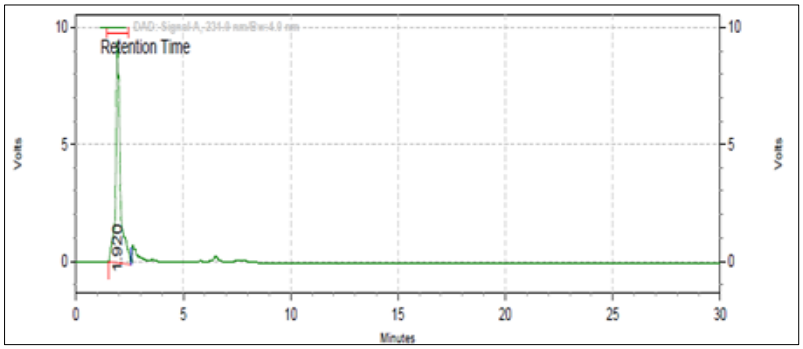

Fig 3: RepresentingC-18 RP-HPLC chromatograms of standard saponin, whole leaf, Gel, Peel, Sap, and Root respectively

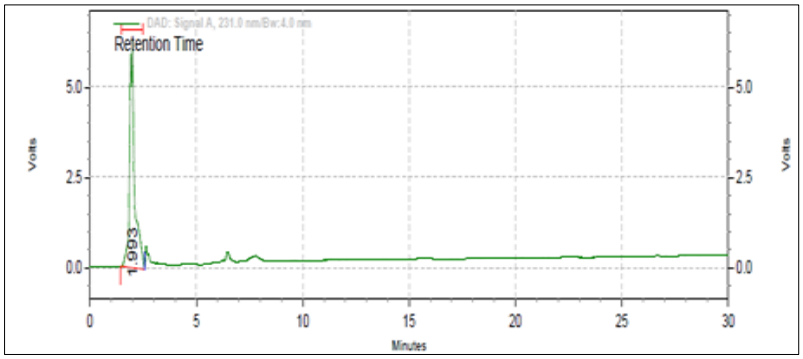

Fig 4: RepresentingC-18 RP-HPLC chromatograms of standard saponin, whole leaf, Gel, Peel, Sap, and Root respectively

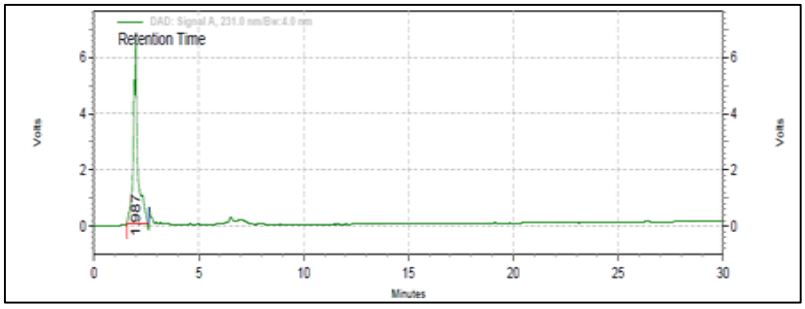

Fig 5: RepresentingC-18 RP-HPLC chromatograms of standard saponin, whole leaf, Gel, Peel, Sap, and Root respectively

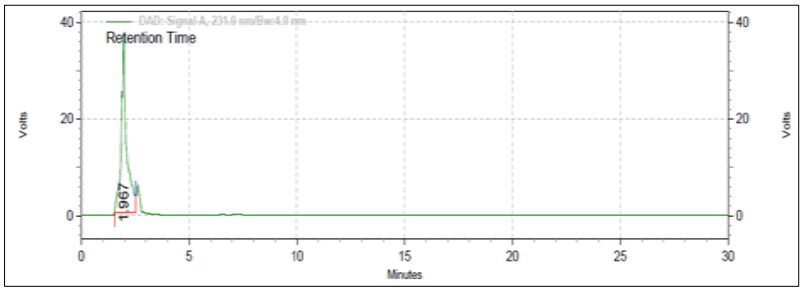

Fig 6: Representing C-18 RP-HPLC chromatograms of standard saponin, whole leaf, Gel, Peel, Sap, and Root respectively

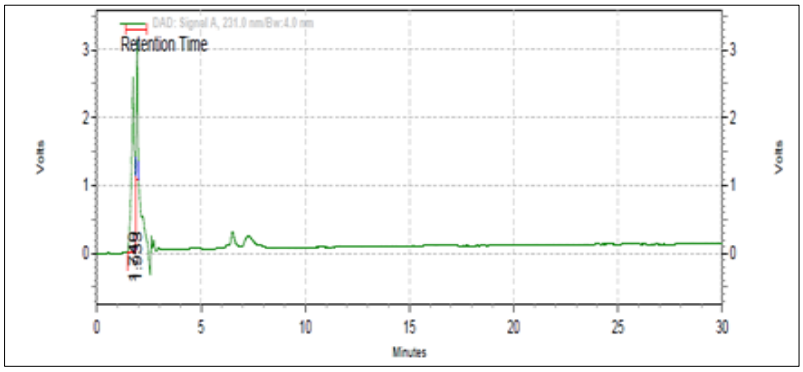

Fig 7: RepresentingC-18 RP-HPLC chromatograms of standard saponin, whole leaf, Gel, Peel, Sap, and Root respectively

Table 1: Concentration of different samples of saponins taken as a standard considering peak area

Sr no	Sample Name	RT	Area	Conc.(mg/g)
1	Sapo-1	1.880	38978115	0.285
2	Sapo-2	1.900	74568165	0.397
3	Sapo-3	1.913	108274102	0. 577
4	Sapo-4	1.913	148766661	0.793
5	Sapo-5	1.920	190916284	1.017

Table 2: Saponins concentration in different tissues in respect of standard considering peak area

Sr no	Name	RT	Area	Conc. (mg/ml)
1	Sapo-3	1.913	108274102	0.577
2	Whole leaf	1.920	18896655	0.101
3	Gel	1.993	13593641	0.072
4	Peel	1.987	12270120	0.065
5	Sap	1.967	77792325	0.414
6	Root	1.933	3835214	0.021

3.3. Characterization of saponins in *Aloe vera* by FTIR

Phytochemical screening of saponins was done by using Fourier Transform Infrared (FTIR) spectrometer. Absorption spectra obtained in the range of 3500-3200 cm-1 show the presence of a hydrogen-bonded hydroxyl group (-OH). In saponin standard and all sample extracts, sharp peaks were seen in the range of 2850 - 3000 cm-1, showing C-H stretching and the carboxylic group. Peaks obtained in the range of 1720.27 to 1734.26 confirm the presence of esters(C=O). Peaks observed at 1600-1630 cm-1 indicated the presence of (C=C) group in the extracted compounds. In all extracts, peaks were observed in the range of 1000-1300 cm-1, which confirms the presence of carbonyl (C-O) and ether (C-O-C) groups. Thus the presence of hydroxyl, carboxylic, esters, and ether functional groups verifies the presence of saponins in *Aloe vera*.

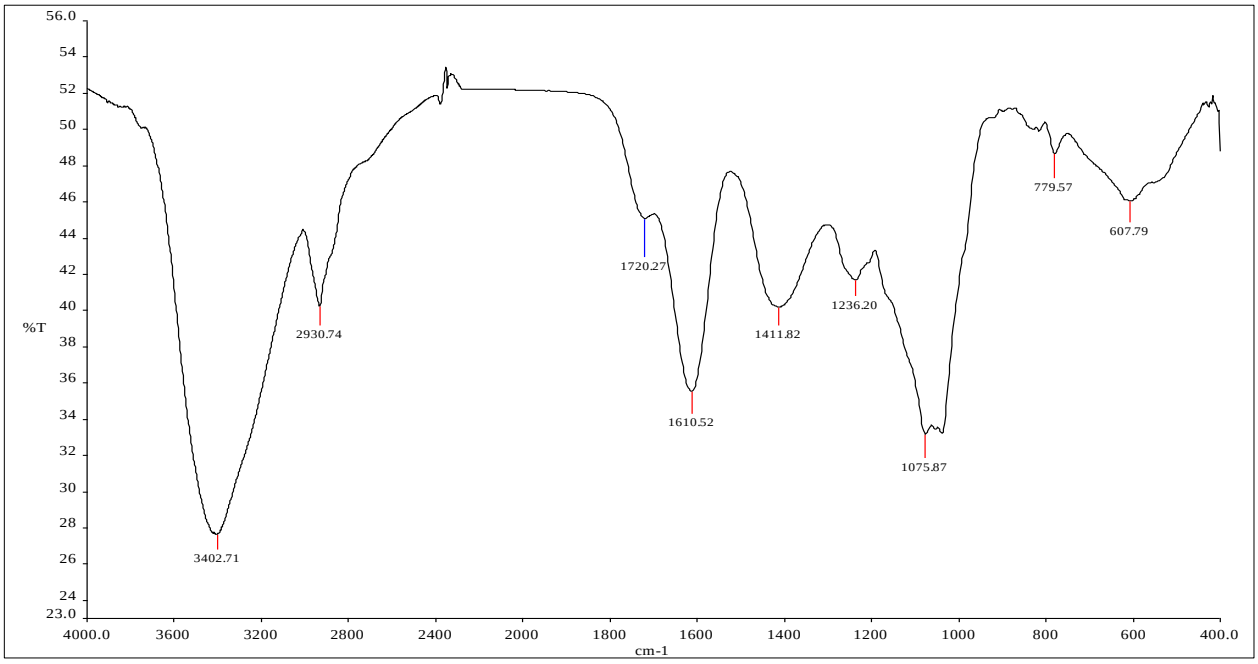

Fig 8: FTIR spectrum for standard saponin

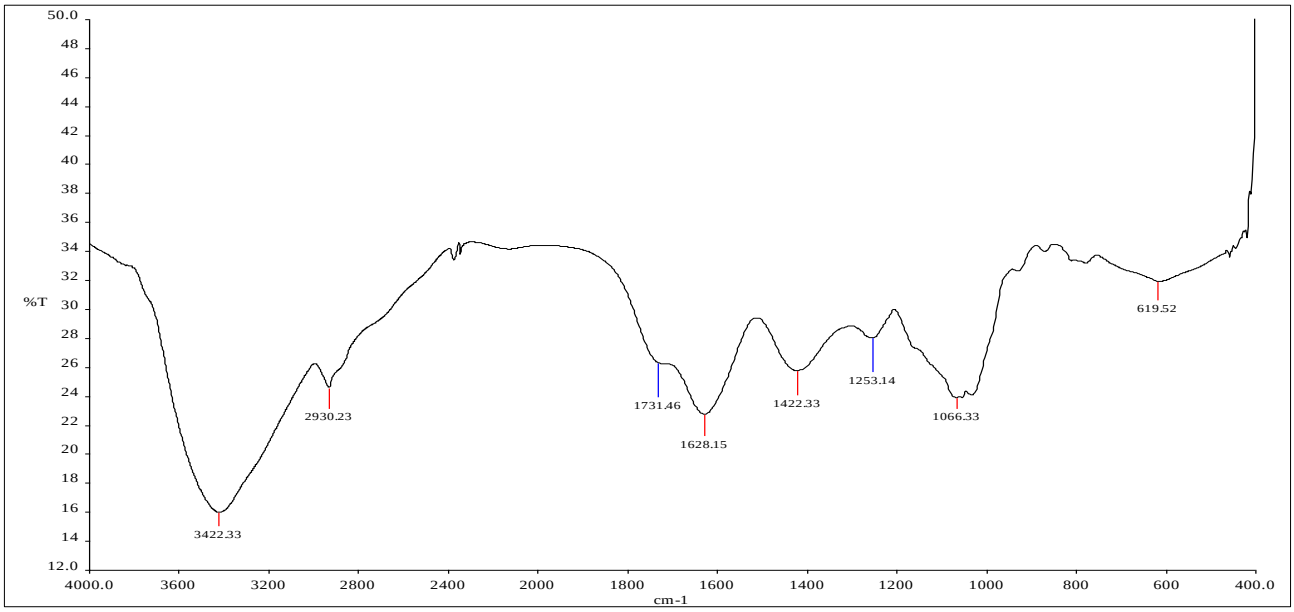

Fig 9: FTIR spectrum for *Aloe vera* whole leaf

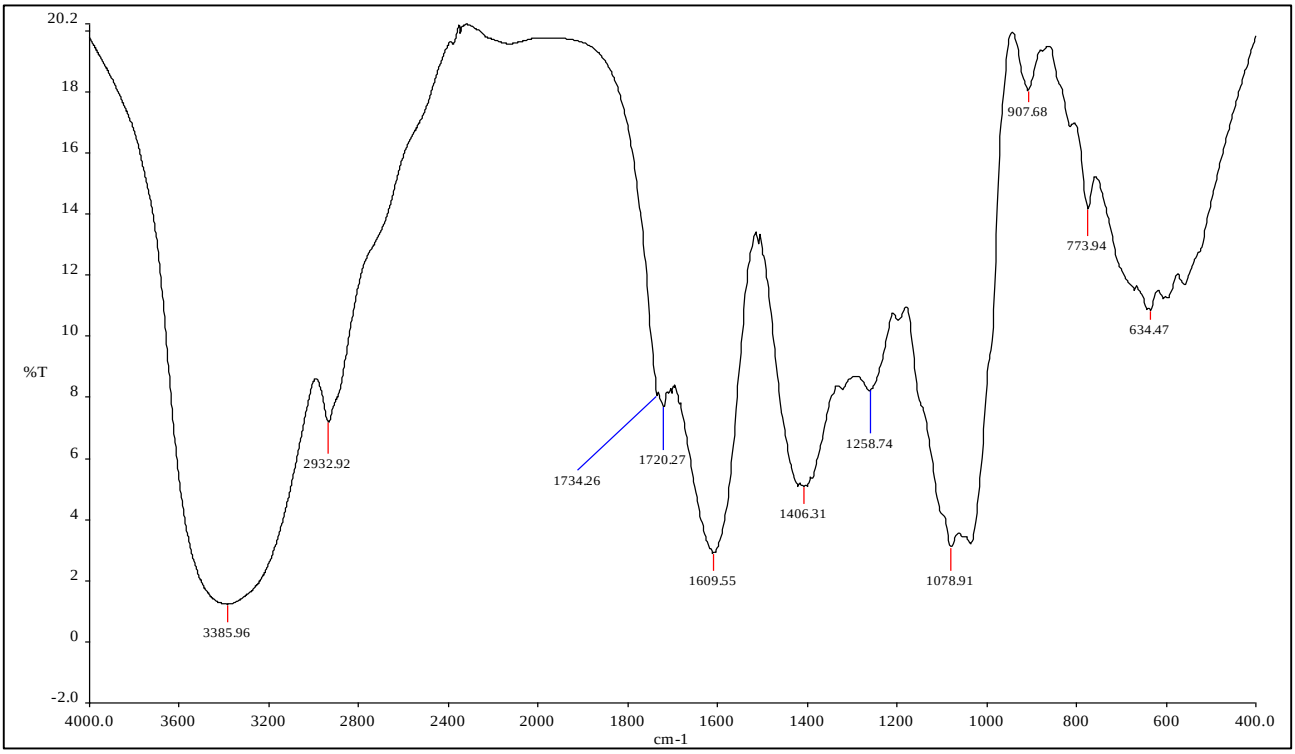

Fig 10: FTIR spectrum for *Aloe vera* gel

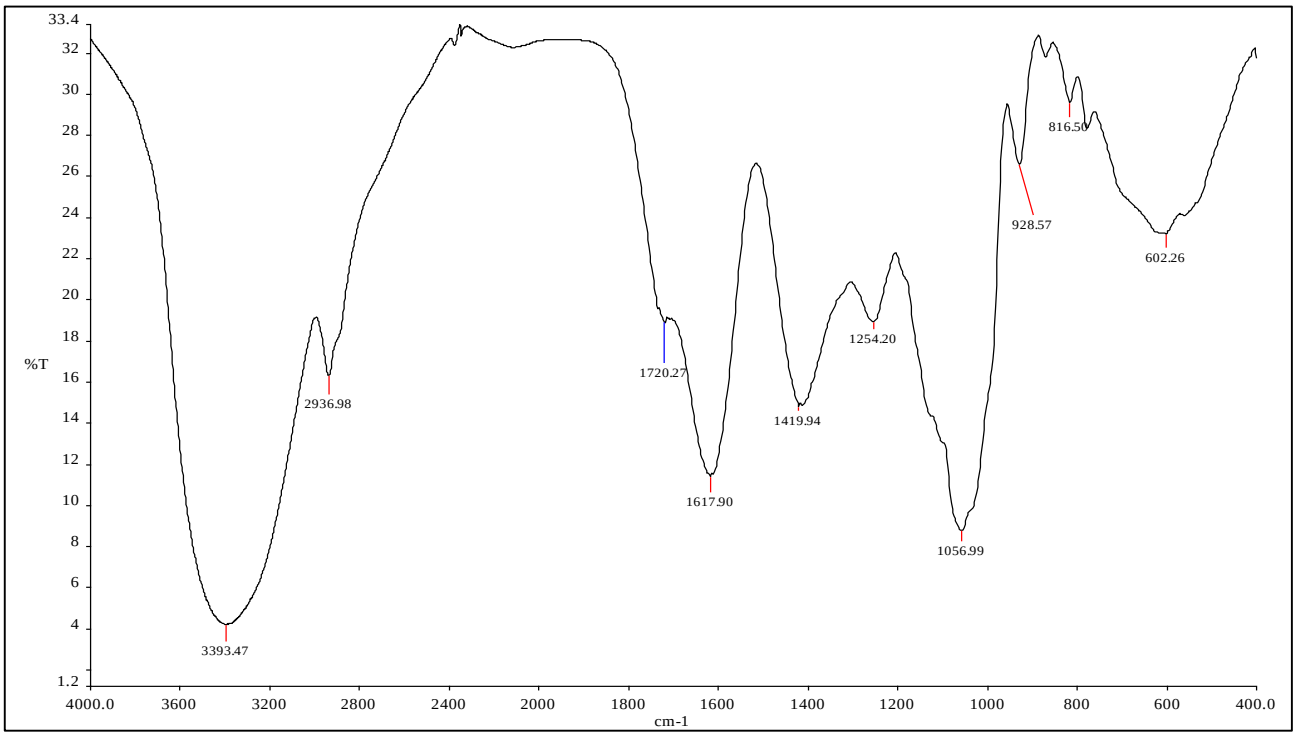

Fig 11: FTIR spectrum for *Aloe vera* leaf peel

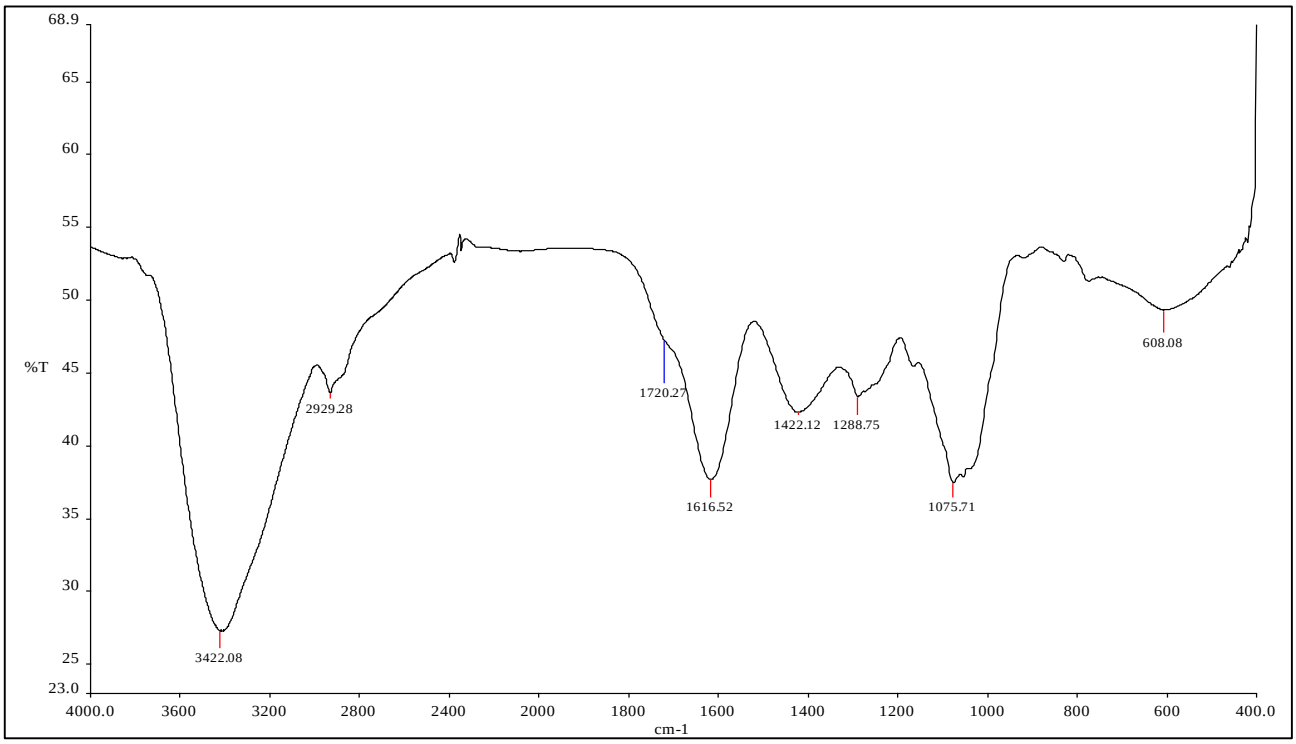

Fig 12: FTIR spectrum for *Aloe vera* sap

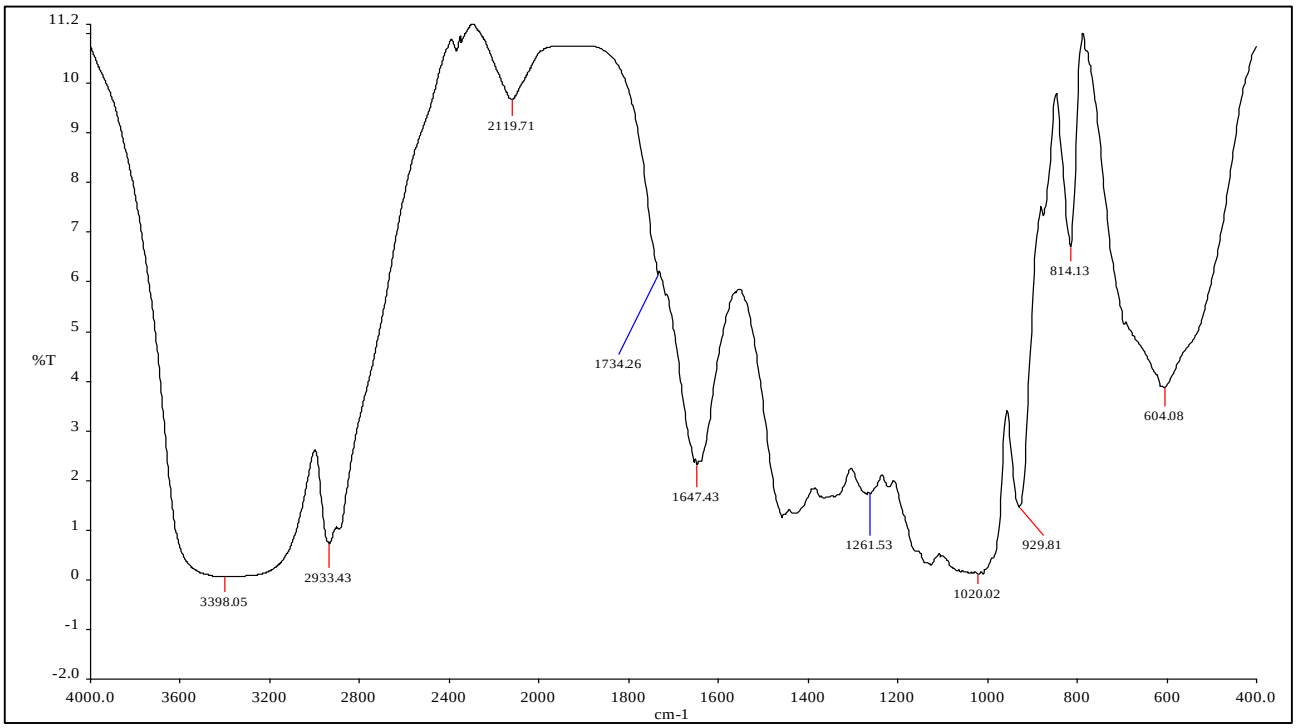

Fig 13: FTIR spectrum for *Aloe vera* root

Table 3: FTIR spectra of tissue-specific extracts of *Aloe vera* (KBr disc), cm-1

Functional Gp.	Saponin Standard	Whole Leaf	Gel	Leaf peel	Sap	Root
-OH	3402.71	3393.85	3385	3393.47	3422.08	3398.05
C-H	2930.74	2928.69	2932	2936.98	2929.28	2933.43
-C=0	1720.27	1720.27	1720.27	1720.27	1720.27	1734.26
C=C	1610.52	1609.32	1609	1617.90	1616.52	1647.43
C-H	1411.82	1420.15	1406.31	1419.94	1422.12	1460.13
C-O	1236.20	1287.66	1258.74	1254.20	1288.75	1261.53
C-O-C	1075.87	1075.91	1078.91	1056.99	1075.71	1127.27

Various techniques exist to extract saponin-rich fractions imitated in different plants. Considerable variation in saponin content might be due to the genotypic variation, ecological conditions of the sampling sites, and methods employed for extraction (Cheok *et al*., 2014) [21]. The conventional extraction techniques are maceration, soxhlet, and reflux extraction, where the green technologies are ultrasound-assisted, microwave-assisted, and accelerated solvent extraction (Heng *et al*., 2013) [22]. The conventional maceration technique practiced here is adopted in 36% of plants for saponin extraction. Simple extraction protocol, easy-to-approach equipment, and utensils requirements have made it a popular choice for researchers (Cheok *et al*., 2014) [21]. RP-HPLC is increasingly used for the detection of saponins due to high sensitivity, accuracy, rapid and straight forward procedures implemented in various plants. By employing a different combination of stationary and mobile phases, better separation of saponins can be affected (Negi *et al*., 2011) [23]. To quantify saponin content in *Aloe vera* whole leaf, gel, leaf peel, sap, and root, RP-HPLC was performed. The goodness of fit of the standard saponin showed good linear regression (r2=0.99) in the range of concentration by using 35% acetonitrile as a mobile phase. The retention time of saponins was observed at 1.9 min, which was also reported in *Chlorophytum borivilianum* (Rashmi Dwivedi, 2013) [24]. Maximum saponin content was observed in sap with 0.414 mg/g and minimum saponin concentration was observed in the root (0.021 mg/g). Large variation in saponins contents might be due to the genotypic variation, ecological conditions of the sampling sites, age, the plants' physiological state, and methods employed for extraction (Cheok *et al*., 2014; Sezgin *et al*., 2010; Kumar *et al*. 2021) [21, 25, 26]. Saponins show different bioactivities, including anti-inflammatory, anti-cancer, and anti-herpes effects (Antunes *et al*., 2003; Gonzalez *et al*., 2003; Ikeda *et al*., 2003; Matsuda *et al*., 2003) [27, 28, 29, 30]. Saponin in *Aloe vera* acts as a natural cleanser and has antiseptic properties (Sparg *et al*., 2004) [31]. These make saponins present in *Aloe vera* an essential natural product, and hence tissue-specific quantification of saponin can be a quick reference for further biochemical research based on saponins present in *Aloe vera*. In the present study, FTIR spectra of all the test samples of *Aloe vera* showed –OH, -C=O, C-H, and C=C absorptions which are a distinct group of saponins. In some other plants like *Moringa oleifera* (moringaeceae) pods, phytochemical screening of saponins was done using FTIR. The IR spectrum of the crude extract showed the presence for hydroxyl group (-OH), carboxylic acids, alkynes, presence of –C=O (esters) and >C-O (ethers), and the ring involvement or aromatic structure of the compound revealed the presence of saponins in the above plant (Sharma and Paliwal, 2013) [17]. In *Entada leptostachya*, FTIR spectra of the test samples showed –OH, -C=O, C-H, and C=C absorptions which are the characteristic of oleanane triterpenoid saponins (Kareru *et al*., 2007) [16]. Thus phytochemical analysis using FTIR confirms the presence of saponins in leaf, gel, peel, sap, and root of *Aloe vera* due to

the presence of characteristics groups of saponins. The present research verifies the presence of saponins in different *Aloe vera* tissues by using High-performance liquid chromatography and Fourier Transform Infrared spectroscopy as described earlier in *Aloe vera* transcriptome and analysis of expression profiles of genes related to saponin metabolism (Choudhri *et al*., 2018) [32].

4. Conclusion

Aloe vera is easily grown in almost every climate, widely distributed, and readily available, but it is necessary to explore biochemical research in *Aloe vera* for proper commercial exploitation of its biological activities. The results of phytochemical analysis of HPLC and FTIR data confirm the presence of saponins quantitatively in different tissues of *Aloe vera* yet not been done previously. Saponins are responsible for the high cosmetic and medicinal value of *Aloe vera* and make it an essential natural product. The results show good sensitivity and accuracy, which can be used as a primary platform for commercializing this bioactive metabolite.

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