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Application of phytochemical screening and a combined FTIR spectroscopy and principal component analysis for effective discrimination of two varieties of *Eclipta alba* (L.) Hassk.

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Abstract : *Eclipta alba* Hassk syn. *E.prostrata* L. and *E.erecta* L., belonging to the family Asteraceae, is a common herb with two types of habit, viz. erect and prostrate. The common name of this herb is karisalnganni or bhringraj. There are four varieties of this herb which are identified with their flowers pink, blue white or yellow. Out of these, the white and yellow varieties are widely used in the medical world and home dishes. They are called as Vellai Karisalanganni and Manjal Karisalangnni based on type of flowers they bearing such as white coloured flowers and yellow coloured flowers, respectively. A literature survey of this plant revealed that many studies have been carried out in this plant in order to characterize or understand their various medicinal properties. For this purpose they have used different parts of this plant without considering the varietal difference. Hence, in the present investigation, an attempt was made to discriminate two varieties of *E. alba* such as White Karisalanganni (WK) and Manjal Karisalanganni (MK) by applying phytochemical screening and a combined FTIR spectroscopy and principal component analysis (PCA). After authentication, leaves of these two varieties were dried and finely powdered. Methanolic and chloroform leaf extracts were prepared using these powders following cold extraction method. After thorough drying, the filtrate of both the extracts were subjected to primary qualitative screening and FTIR analyses. Then, FTIR spectral data were subjected to PCA. In FTIR analysis, both the varieties are differed from each other in specific functional groups. MK variety only shows C=H and C=O stretches which represent the presence of functional groups of alcohol and ester, respectively. Chloroform extract of WK variety only shows alkyne group. To summarize, both the varieties are chemically differed from one another very slightly based on their score plots and also their Eigen value percentage of variance when FTIR data subjected to PCA analysis. Thus, phytochemical screening and a combined FTIR spectroscopy and PCA are used for effective discrimination of both the varieties of this plant. The result of this investigation will facilitate proper authentication of these two varieties for research purpose as well as for food and pharmaceutical applications.

Keywords : *Eclipta alba*, Phytochemical screening, FTIR spectroscopy, PCA analysis, Varietal difference, Methanolic extract, Chloroform extract.

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Introduction

Eclipta alba Hassk syn. *E.prostrata* L. or *E.erecta* L., belonging to the family Asteraceae, is a common herb found in the tropical and subtropical regions of the world during rainy season. It grows well in moist places and shows two types of habit, viz. Erect and prostrate; hence they called as *E.erecta* and *E.prostrata*, respectively[1]. It has a short flat or round stem with white flowers or yellow flowers on a long stalk. The leaves are opposite and lance shaped. The roots are well grown cylindrical and dark brownish or grayish in color. Floral heads are solitary, achene compressed narrowly winged and 5-8 mm in diameter. There are four varieties of this herb which are identified with their flower colour such as pink, blue, white or yellow[2]. Out of these, herbs with the white and yellow flowers are widely used in the medical world and home dishes. They are called as Vellai Karisalanganni and Manjal Karisalanganni based on type of flowers they bearing such as white coloured flowers and yellow coloured flowers, respectively (Fig.1A and 1B). It is a valuable medicinal herb reported for its various pharmacological and biological activities like hepatoprotective[3], hair growth promotion[4], antimicrobial property[5], anticancerous activity[6] and antidiabetic activity[7].

Fig. 1A - *Eclipta alba*- plant with white flower- Vellai Karisalanganni (WK).
Fig. 1B - *Eclipta alba*- plant with yellow flower- Manjal Karisalanganni (MK)

Traditional and folklore medicines play an important role in health services around the Globe. About three quarters of the World's population relies on plants and its extracts for health care[8]. India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants. The active biocomponents in medicinal plants are defined as chemical compounds which directly or indirectly prevent or treat disease[9]. The most important of these bioactive chemical constituents of plants are alkaloids, tannins, flavonoids and phenols. These chemical compounds have countless benefits to humans, which are exploited as natural pesticides, flavors, fragrances, fibers, beverages and also used as a precursor for various therapeutic drugs[10]. The medicinal worth of plant lies in their phytochemical constituents which help to induct different physiological effects on human body. Therefore, through phytochemical screening one could detect the various important bioactive compounds of the interesting plant which may be used as the bases of modern drugs for curing various diseases[11].

FTIR measures the vibrations of bonds within chemical functional groups and generates a spectrum that can be regarded as a metabolic "fingerprint" of the sample. By attaining IR spectra from plant samples, it might possible to detect the minor changes of primary and secondary metabolites[12,13]. At present, particularly in phytochemistry, FTIR has been applied to identify the concrete structure of certain plant secondary metabolites[14,15]. FTIR spectroscopy has been used in conjunction with PCA to detect and classify a broad range of cell-wall mutants from a large mutagenized *Arabidopsis* population[16]. PCA is one of the most common multivariate statistical techniques to detect minute variations between two or more samples. PCA reduces the dimensionality of the original data set by calculating a new set of variables known as Principal Components. These Principal Components (PC's) are calculated from linear combinations of the original variables and are organized according to the amount of variance in the original data set that they explain which are uncorrelated to one another. Typically, only the first two PC's are required to explain the majority of the variance (80 – 90%) in the original dataset and are used to generate a graphical output called a score plot. In the score plot, the samples are arranged in space relative to each other according to their first two PC scores. The samples with

similar scores will occupy similar position; whilst those with dissimilar scores will be positioned some distance away, thus allowing clusters to be identified[17,18,19,20].

A literature survey of this plant revealed that many investigations have been carried out in this plant in order to characterize or understand their various medicinal properties. For this purpose they have used different parts of this plant without considering the varietal difference. For example, infrared and energy dispersive X-ray spectra of different parts (leaf, stem, and root) of this plant were recorded[21]. Physicochemical and phytochemical properties of aerial parts of this plant were evaluated using ethanol, petroleum ether, benzene, chloroform and aqueous solvents[22]. *E.alba* plants are proved as an important source of many bioactive compounds through various pharmacological activities [23]. The chemical compositions of methanolic extract of this plant leaves was investigated using GC-MS technique[24]. In addition to phytochemical screening, antioxidant potential of this whole plant has been evaluated[25]. Recently, *E.prostrata* leaves were characterized without mentioning variety name using FTIR spectroscopy, CHNS (carbon, hydrogen, nitrogen and sulphur elemental analyser) and ICP-MS (inductively coupled plasma mass spectrometry) techniques and proved that this plant has some bioactive constituents and may be useful for isolation of pharmacologically important compounds[26]. None of the researchers mentioned the variety of this plant i.e either plant with white flower or plant with yellow flower was used for their studies. So far, there is no published reports on varietal difference of this species by applying any technique. Hence, in the present investigation, an attempt was made to discriminate two varieties of *E. alba* such as White Karisalanganni (WK) and Manjal Karisalanganni (MK) using qualitative phytochemical screening and FTIR spectroscopy analysis. Furthermore, FTIR data was subjected to PCA in order to find out chemical variations between these two varieties.

Materials and Methods

Plant collection, authentication and processing

Leaves of two varieties of *E. alba* were collected locally from different places of Coimbatore, Tamil Nadu, India. The plant samples were authenticated by Botanist, Department of Botany, Bharathiar University, Coimbatore. The collected samples from various places were pooled and made into one composite sample for each variety. The composite samples were then washed thoroughly under running tap water to remove dust particles and oven dried at 40^{o}C for 48 hours.

Plant extraction and estimation of plant extractive yield

For extraction of plants, cold extraction method was followed. In this protocol, the dried plant samples were ground into fine powder using mixer-grinder. The plant powder weighing 25 g was placed separately in conical flasks containing 100 ml methanol and 100 ml chloroform and flasks were kept at room temperature for 48 hours under shaking (100 rpm). After two days, the extracts were filtered using Whatman filter paper No.1 and collected filtrate was allowed to dry separately at room temperature in the petridishes. Plant extractive yield (W/W) of methanolic and chloroform extracts was calculated using this formula W_2-W_1/W_0X100. Where W_2 is the weight of the extract and the container, W_1 the weight of the container alone and W_0 the weight of the initial dried sample.

Sample extract preparation, primary qualitative phytochemical screening and FTIR analysis

One gram of plant extract was dissolved in 10 ml of respective solvent to make the stock solution of 100 mg/ml. For primary qualitative analysis aliquots containing 1mg/10µl was taken from this stock and was diluted further as per experiment requirements. Qualitative phytochemcial screening using methanolic and chloroform extracts for the identification of various secondary metabolites such as alkaloids, flavonoids, steroids, terpenoids, anthroquinones, phenols, saponins, tannins, cardiac glycosides, pholobatanins and coumarins were performed as per standard procedures[27,28]. For FTIR analysis, 10 mg of the dried extract powder was encapsulated in 100 mg of KBr and the resulting pellet was placed in FTIR spectroscope and analyzed with a scan range from 500 to 4000 cm-^{1} with a resolution of 4 cm-^{1} .

Principal Component Analysis

Functional groups analyzed through FTIR using both the extracts obtained from two varieties of *E. alba* were subjected to PCA to find out chemical variations between two varieties using Past3 software.

Results and discussion

Plant extractive yield

In the present study, methanolic extract showed maximum percentage of yield i.e 7% for WK variety and 6% for MK variety. Whereas, chloroform extract showed 5% yield for WK variety and 4% for MK variety (Table 1). When compared to chloroform solvent, the percentage yield extract of methanol was found to be slightly higher. It indicates that the extraction yield increases whenever solvent with increasing polarity used in plant extraction. Extraction efficiency is affected by the chemical nature of phytochemicals, the extraction method used, sample particle size, the solvent used as well as the presence of interfering substances[29].

Table 1: The percentage yield extracts of two varieties of *E. alba* i.e white karisalanganni (WK) and manjal karisalanganni (MK).

Name of the extract	Yield (%)	
	WK	MK
Methanolic extract	7	6
Chloroform extract	5	4

Primary qualitative phytochemical screening

In primary qualitative phytochemical screening, all the secondary metabolites such as alkaloids, flavonoids, steroids, terpenoids, anthroquinones, phenols, saponins, tannins, cardiac glycosides, pholobatannins, coumarins were found to be present in methanolic extract of WK variety where as flavonoids, anthroquinones, saponins and pholobatannins were found to be absent in the same extract of MK variety. Chloroform extract of WK variety showed the occurrence of some secondary metabolites such as alkaloids, flavonoids, phenols and coumarins. Only alkaloids and phenols were found in the chloroform extract of MK variety (Table 2). The phytochemicals screening tests serves as the preliminary step in predicting the various types of potential phytocompounds[30]. In the present study, the methanolic extract of *E.alba* WK variety has shown the presence of maximum number of phytochemicals compared to MK variety. Chloroform extracts of these two varieties showed less number of phytocompounds. Both the extracts of WK variety showed positive result for flavonoids presence whereas MK variety gave negative result. This is one of the important observation of this investigation.

Table 2: Phytochemical screening of methanol and Chloroform leaf extracts of two varieties of *E. alba* i.e Vellai Karisalanganni (WK) and Manjal Karisalanganni (MK).

Sl.No	Phytochemicals	Methanol Extract		Chloroform Extract	
		WK	MK	WK	MK
1.	Alkaloids	+	+	+	+
2.	Flavonoids	+	-	+	-
3.	Steroids	+	+	-	-
4.	Terpenoids	+	+	-	-
5.	Anthroquinones	+	-	-	-
6.	Phenols	+	+	+	+
7.	Saponins	+	-	-	-
8.	Tannins	+	+	-	-
9.	Cardiac glycoside	+	+	-	-
10.	Pholobatannins	+	-	-	-
11.	Coumarins	+	+	+	-

(+) Indicate the present, (-) Indicate the absent

Previous phytochemical studies in this species showed that methanolic extract of whole plant yielded eleven bioactive compounds[31]. Similar to our result, positive result for flavonoids was obtained when chloroform extract of aerial parts of *E.alba* was subjected to qualitative phytochemical analysis[22]. Similarly, Positive signal for flavonoids was obtained when whole plant methanolic extract was screened for the presence of phytoconstituents by Sinha and Raguwanshi[25]. These observations indicate that they might have been used WK variety for their studies. Flavonoids and phenolic acids make up one of the most pervasive groups of plant phenolics. These components function as reducing agents, free radical scavengers and quenchers of singlet oxygen formation. In addition, they play an important roles in the control of cancer and other human diseases[32].

FTIR analysis

Results of the FTIR spectra of methanolic and chloroform extracts of WK and MK varieties of *E.alba* revealed the presence of different functional groups (Table 3). The FTIR spectrum analysis of methanolic extract of WK (Fig. 2) confirmed the presence of different functional groups with a peak values for alkane at 2849.30 cm^{-1} and 2947.66 cm^{-1}, alkene at 1645.94 cm^{-1}, aromatic phenol at 1463.70 cm^{-1}, amine at 1157.08 cm^{-1} and 1347.99 cm^{-1} and halo compound at 669.17 cm^{-1}. The observed peaks in methanolic extract of MK at 2857.98 cm^{-1} (C-H strong stretch) and 1376.92 cm^{-1} (C-H bending stretch) correspond to presence of alkane. The peaks at 1428.02 cm^{-1}, 1145.50 cm^{-1} and 656.64 cm^{-1} are due to aromatic phenol, amine and halo compound, respectively, present in the methanolic extract of MK. The chloroform extract of WK (Fig.3) showed peaks at 2857.02 cm^{-1}, 2924.52 cm^{-1} and 1367.28 cm^{-1} which represent the presence of functional group of alkane. The peak found at 1907.25 cm^{-1} indicates the occurrence of alkyne (C=C=C stretch) in chloroform extract of WK. Peaks obtained at 1557.23 cm^{-1} and 836.95 cm^{-1} indicated the presence of aromatic phenol and halo compound, respectively. C-N stretch obtained at 1165.75 cm^{-1} and 1276.64 cm^{-1} using this extract denotes the presence of amine. In chloroform extract of MK, alkene is represented by peaks at 2924.52 cm^{-1} and 1364.39 cm^{-1}. The peaks noted at 2775.06 cm^{-1} represents the presence of functional group of alcohol. The presence of phenol and halo compound are confirmed by peaks at 1436.70 cm^{-1} and 808.99 cm^{-1}, respectively. Peaks obtained using this extract at 1245.78 cm^{-1} and 1319.07 cm^{-1} represent the presence of amine.

Table 3: FTIR peak values and functional groups of methanol and chloroform leaf extracts of two varieties of *E. alba* i.e Vellai Karisalanganni (WK) and Manjal Karisalanganni (MK).

Sl.No.	Functional groups	Bonds	Peak values				
			Group frequency wavenumber cm^{-1}	Methanol Extract		Chloroform Extract	
				WK	MK	WK	MK
1.	Alkane	C-H Stretch (Strong) C-H bending (Variable)	2840-3000 1480-1350	2849.30 2947.66 -----	2857.98 1376.92	2857.02 2924.52 1367.28	2924.52 1364.39
2.	Alkene	C=C Stretch	1680-1630	1645.94	1642.08 1645.94	-----	-----
3.	Alcohol	C-H (Medium)	2830-2695	-----	2753.85	-----	2775.06
4.	Alkyne	C=C=C Stretch	2000-1900	-----	-----	1907.25	-----
5.	Ester	C=O (Strong)	1740-1720	-----	-----	------	1728.87
6.	Aromatic phenol	C=C Stretch (Medium – Weak	1600-1400	1463.70	1428.02	1557.23	1436.70
7.	Amine	C-N Stretch (Medium-Weak)	1360-1080	1157.08 1347.99	1145.50	1165.75 1276.64	1245.78 1319.07
8.	Halo compound	C-Br	900-600	669.17	656.64	836.95	808.99

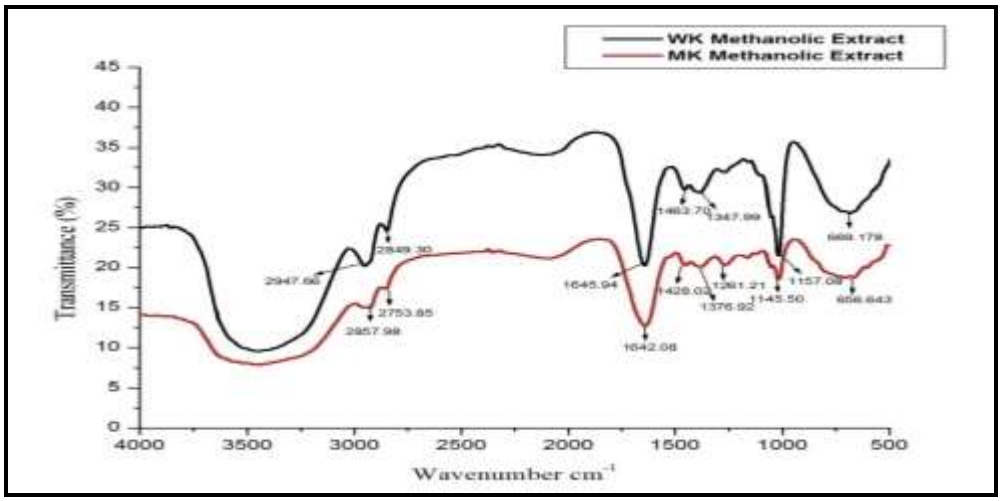

Fig. 2. FTIR spectra of methanolic leaf extract of both varieties of *E. alba.*

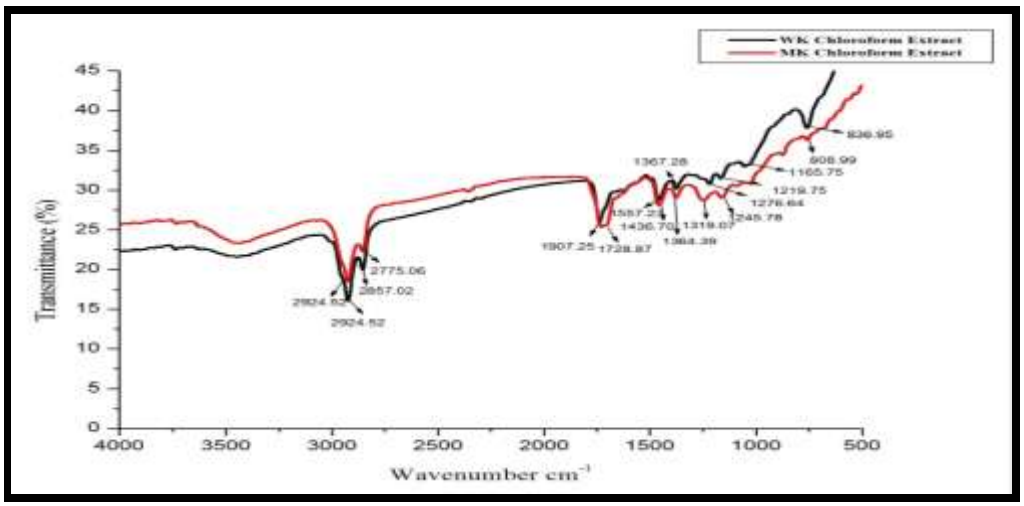

Fig. 3. FTIR spectra of chloroform leaf extract of both varieties of *E. alba.*

The FTIR spectroscopy has been used to identify the functional groups of the active components present in the plant extracts based on peaks values in the region of IR radiation. When the extract was passed through the FTIR, the functional groups of the components are separated based on its peaks ratio[33]. The results of FTIR analysis of methanolic and chloroform extracts of WK and MK varieties of *E.alba* confirmed the presence of alkane, alkene, aromatic phenol, amine and halogen compound in common (Table 3). The presence of these functional groups in this plant extracts exhibited the occurrence of alkaloids in maximum quantity[34]. This is also proved in primary qualitative phytochemical screening of the present study where both the extracts of the both the varieties have shown positive results for alkaloids. When analyzed the spectral features of methanolic and chloroform extracts of WK and MK, it was observed that functional group of alcohol was found only in MK variety of both the extracts and ester group was found only in chloroform extract this variety. This observation is in agreement with the earlier report of Anand et al., (2014)[24] who reported the presence of various potential bioactive components including methyl ester, butyl octyl ester, eicosyl ester in methanolic leaf extract of *E.alba* using GC-MS. The identified ester compounds in this plant has shown many medicinal properties such as anti-inflammatory, cancer preventive, dermatitigenic, hypochlesterolemic and anemiagenic insectifuge[35]. Alkyne group was present only in chloroform extract of WK variety. The same observation was made by Muruganantham et al., (2009)[21]. From this investigation, it is clearly understood that both the varieties of *Eclipta alba* i.e WK and MK are differed from one another in some of the phytoconstituents which is also confirmed by phytochemical screening and FTIR analysis. The difference in the plant components might arise from several environmental (climatic, seasonal and geographical) and genetic factors which determine the medicinal properties of plants [36,24].

Principal Component Analysis

The data of functional groups analyzed by FTIR were subjected to PCA. Using this statistical tool, functional groups obtained using methanolic extract, a PCA score plot has been drawn and represented in Fig.4. It was noted from the plot that some functional groups such as alkane, alcohol and halo compound are positioned some distance away from central axis which indicate they may have dissimilar scores. Functional groups of aromatic phenol and amine are positioned somewhat nearer to the central axis and alkene, alkyne and ester are positioned very close to or on the central axis. These observations denote that these functional groups have almost similar scores.

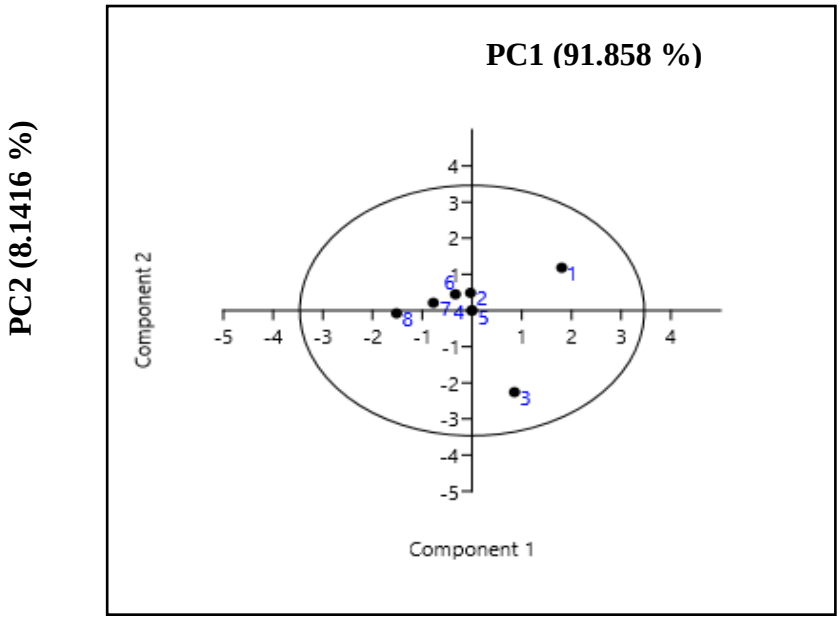

Fig. 4. PCA score plot (PC1XPC2) for functional groups of methanolic extract.

Principal Component 1 (PC1) - Methanolic extract of WK variety
Principal Component 2 (PC2) - Methanolic extract of MK variety

- **1-Alkane**
- **2-Alkene**
- **3-Alcohol**
- **4-Alkyne**
- **5-Ester**
- **6-Aromatic phenol**
- **7-Amine**
- **8-Halo compound**

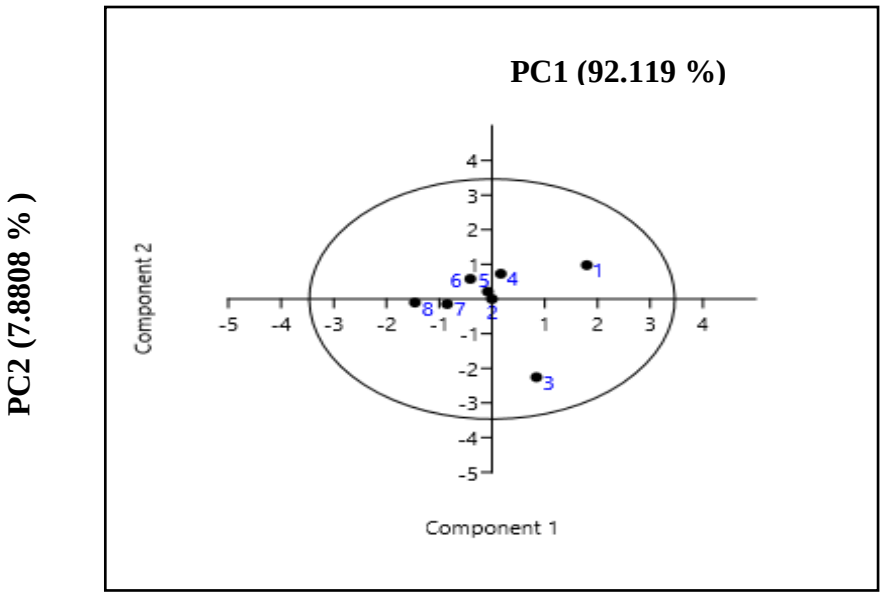

Fig. 5. PCA score plot (PC1XPC2) for functional groups of chloroform extract

Principal Component 1 (PC1) - Chloroform extract of WK variety Principal Component 2 (PC2) - Chloroform extract of MK variety

- **1-Alkane**
- **2-Alkene**
- **3-Alcohol**
- **4-Alkyne**
- **5-Ester**
- **6-Aromatic phenol**
- **7-Amine**
- **8-Halo compound**

Functional groups obtained using chloroform extract, a PCA score plot has been drawn and illustrated in Fig.5. Similar to methanolic extract score plot, functional groups such as alkane, alcohol and halo compound obtained using chloroform extract are positioned some distance away from central axis. The remaining all the functional groups such as aromatic phenol, amine, alkene and ester are positioned very nearer to or on the central axis except alkyne group which was positioned at medium distance away from the central axis. Even though all the eight functional groups of these two plant varieties exhibit various scores, but Eigen value percentage of variance obtained for both the extracts of these varieties indicate that there are only slight chemical variations in their phytoconstituents. Eigen value variance percentage was found to be 91.858 and 92.119 for methanolic and chloroform extracts of WK variety, respectively. It was 8.1416 and 7.8808 for methanolic and chloroform extracts of MK variety, respectively. These slight chemical variations are also confirmed by qualitative phytochemical screening and FTIR analysis in the present investigation. This type of a combined FTIR and PCA approach was applied to lignified softwood cell walls in order to test the potential of this method to give information on polysaccharide composition[37]and to study changes in the cell wall and cellulose content of developing cotton fibers[38].

Conclusion

In conclusion, both the varieties of *E.alba* are discriminated from each other in the presence or absence of some of the phytoconstituents by primary qualitative screening and a combined FTIR-PCA. Both the extracts of WK variety showed positive result for flavonoids presence whereas MK variety gave negative result. This is one of the important observation of this investigation. In FTIR analysis, both the varieties are differed from each other in specific functional groups such as alcohol, ester and alkyne. Only MK variety has shown alcohol and ester groups and only WK variety has alkyne group. Phytochemical and FTIR observations are also confirmed by PCA which indicates both the varieties are chemically differed from one another very slightly based on their score values and also their Eigen value percentage of variance. The presence of various phytocompounds i.e. eleven in WK variety and seven in MK variety justifies the usage of leaves of both the varieties of *E.alba* for treatment of various diseases by traditional practitioners. Thus, phytochemical screening and a combined FTIR-PCA are used for discrimination of both the varieties of this plant effectively. The result of this investigation will facilitate proper authentication of these two varieties for research purpose as well as for food and pharmaceutical applications.

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