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Fourier transform infra-red spectrochemical analyses of Pieridae butterfly wings

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ABSTRACT: Fourier transform infrared (FTIR) spectroscopic technique was carried out in wings of nine different Pierid butterflies to deduce the functional groups and properties of cuticular hydrocarbons which play a pivotal role in exhibiting charismatic colour patterns, controlling body temperature, attracting potential mates and camouflaging against predators. There were three major types of hydrocarbons present in all butterfly wings including alkanes, alkenes and methyl hydrocarbons. The second predominant compounds found in the butterfly wing region include alkyl halides, alcohols and phenols. There were no significant differences in the functional groups among the wings of butterflies. FTIR analysis of nine different Pierid butterfly wings showed many relative sizes of peaks. There were no significant differences in the chemical composition of wings but the colour differences were observed among the nine different butterflies. It was inferred that not majorly due to the differences in the cuticular hydrocarbons and may be due to the presence of different microstructures like scales, ridges and grooves.

KEY WORDS: Cuticular hydrocarbons, microstructures, functional groups, pterins

INTRODUCTION

Butterflies are celebrated widely because of their aesthetic appeal and are often considered as a flagship group of insects in the field of insect biology and conservation (Aarti and Arya, 2021; Kunte *et al*., 2020). The majority of butterflies have pigmented phenotypes. The color patterns exhibited among butterflies are classified as structural colors and pigmented colors (Kaspar *et al*., 2019). Colorations of butterfly wings are due to the presence of microstructures and specific pigments. These pigments appeared colorful because they absorb and reflect light in a certain wavelength, and the remaining is dissipated as heat (Sharmila *et al*., 2020; Shamim *et al.*, 2014). This phenotypic plasticity among butterflies has led to many interesting studies. Butterfly wings are intensively studied by many researchers because of their charismatic colour, hydrophobic behaviour, wettability, fluorescence, self-cleaning property, and also for their flexibility and lightness (Devi *et al*., 2021, Krishna *et al*., 2020, Kaspar *et al*., 2019).

Pieridae is one of the largest families under Lepidioptera which consist of more than 1100 species. Pierid butterflies exhibit sexual dichroism (Giraldo and Stavenga, 2006).Incident light on the scale creates a thin film interference, which changes the colour of the reflected light. Based on the information gained from the Atomic Force Microscopic images of scales, it is known that colour of the reflected light depends upon the periodic arrangements of ridges (Kaspar *et al*., 2019).

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Scanning Electron Microscopic analysis of yellow and white wing scales of Pierid butterflies has shown the presence of densely decorated ovoid beads in their cross ribs (Giraldo, 2008). The presence of beads on the cross ribs is one of the general characteristics of Pierid butterflies.

Fourier transform infrared (FTIR) spectroscopic technique can be used to study the spectrochemical analysis of diverse materials ranging from small molecules to supra molecular structures (Xu and Gowen, 2021).Structural features can be analyzed by using FTIR which is based on the inter and intra molecular interactions of functional groups present in that molecule in all aggregation states (Kannan *et al.*, 2020; Kamnev et *al.*, 2021). Many studies using FTIR have been done on biological samples such as live cells, tissue samples, body fluids (Baker *et al*., 2014), and also in tissues of certain disorders like myopathies and brain tumours (Petibois *et al*., 2009), body fluid traces (Mistek and Lenev, 2018). The biochemical information provided by FTIR will be used for diagnosis and forensic purposes. Only few studies of FTIR have been done on arthropods like honeybees, dragonflies (Machovic *et al*., 2017). Very few reports are available on FTIR analysis in butterflies (Tian *et al.*, 2015, Krishna *et al*., 2020). An attempt has been made in the spectrochemical analysis of various functional groups present in the wings of different Pierid butterflies by using the FTIR technique.

MATERIALS AND METHODS

Butterflies of the family Pieridae were collected from four regions around Madurai, Tamilnadu, India namely Alagar Hills Reserve Forest (10°04’39.0"N; 78°12’60.0"E), Sirumalai Hills Reserve Forest (10°11’01.1"N; 77°59’49.9"E), Kiluvamalai Reserve Forest (10°04’18.1"N; 78°10’11.0"E) and The American College campus (9°55’46.1"N; 78°08’01.3"E). The specimens of Pierid butterflies were collected with the help of insect net and hand picking during the month of December 2018 to September 2019. The collected specimens were stretched on stretching board, and then transferred to the insect boxes. The collected butterflies were preserved by mixing phenol and camphor (3:1 ratio). The cotton was dipped in the phenol- camphor mixture and kept in four corners of the insect box. Identification was done on the basis of literature and keys. Identified species of Pierid butterflies were labelled properly. Nine species of Pieridae butterflies were selected for the study which included *Eurema brigitta*, *Catopsilia pyranthe*, *Colotis danae***,** *Pareronia valeria*, *Appias lyncida*, *Pareronia ceylanica*, *Colotis etrida*, *Eurema hecabe* and *Ixias pyrene*.

The study focused on spectrochemical analysis of Pierid butterflies by using FTIR technique. The forewings of nine butterflies were chosen for this study. The functional groups in the wings were identified by Fourier Transform Infrared spectroscopy (FT-IR Model- Thermo fisher 380). The biomolecules in butterfly wings are characterized at room temperature. The FTIR spectrum was obtained in the mid IR Region of 400 – 4000 cm^{-1}. With potassium bromide crystals, the wings of the butterflies were directly placed and the spectrum was recorded (Sackey *et al.*, 2018). The recorded readings were plotted in the graph using Origin 2021b software (version - 9.85).

RESULTS AND DISCUSSION

The spectrochemical analyses of different regions of nine Pierid butterflies revealed the cuticular hydrocarbons. The selected wing regions of nine Pierid butterflies for FTIR analysis includes apical forewing region of *C. nerissa*, basal forewing region of *C. pyranthe*, discal forewing region of *P. valeria*, basal forewing region of *P. ceylanica*, apical forewing region of *C. danae*, discal forewing region of *E. hecabe*, discal forewing region of *A. lyncida*, basal forewing region of *C. etrida* and discal forewing region of *I. pyrene*. Different regions of wings were selected based upon their different colors. (Table 1; Fig. 1, 2, 3, 4 and 5). FTIR measurement of nine different butterflies of the Pieridae family was carried out to find the chemical composition spectra.

Alkanes are saturated hydrocarbons, characterized by absorption due to C-H Stretching and bending. The structure of n-alkane with a longer hydrocarbon chain allows the close packing of molecules that acts as a barrier to water and well suited for hydrophobic property (Drijfhout *et al.*, 2009). Alkanes are the most predominant hydrocarbon present in all the selected butterflies. In the present study, in *C. nerissa*, the observed absorbance spectra bands at 1339, 2967, 2915, 2836, 1374 and 1339 cm^{-1} were assigned to be alkanes. The absorbance spectral values of alkanes in P. valeria correspond with 2959, 2847, 1464 and 1377 cm^{-1}. In *P. ceylanica,* the absorbance spectra band at 2966, 2901, 1436, and 1351 cm^{-1} were assigned to be alkanes. In *C. danae,* the spectra bands at 2953 and 2919 cm^{-1} correspond with alkanes. In *E. hecabe,* the absorbance spectra bands at 2949, 2924, 2861 and 1365 cm^{-1} represent alkanes. Spectral peak value of alkanes in *A. lyncida* is 2919 and 1370 cm^{-1}. In *C. etrida*, the observed bands at 1382 cm-1 belong to alkanes. In *I. pyrene,* the spectral peak value for alkanes was observed at 2970, 2919, 2842 and 1374 cm^{-1}.

The outer surface layer of cuticle is composed of a matrix of alkenes into which n-alkanes are embedded. Alkenes have three structural features which include length of the long hydrocarbon chain, either sides of the double bond and the angle formed by the double bond. This structural complexity renders the molecule more specific (Rundel *et al*., 2005). In *C. nerissa,* the absorbance band at 1645 cm^{-1} was assigned to be alkenes. The alkenes group has C=C stretch. In *C. pyranthe*, the observed absorbance spectra bands at 1800 and 1647cm^{-1} belong to alkenes. These are characterized as C=O and C=C stretch. In *C. danae*, the observed band is at 1655 cm^{-1} represent alkenes. In all other selected butterflies alkenes were absent.

In *P. valeria*, the absorbance spectra band at 3133cm^{-1} represent alkynes. In *C. etrida*, the observed absorbance spectra bands at 3363 and 3235cm^{-1}correspond to alkynes (terminal). In *I. pyrene*, the band at 3227cm^{-1} represent alkynes (terminal). These are characterized as Ca"C-H, C-H stretch. These are terminal alkynes with strong, narrow bands. This can be an important diagnostic tool because very few organic compounds show absorption in this region. The mobility of these compounds (alkanes, alkenes and alkynes) is important for its functions, for example in wasps, the state of outer layer (solid or liquid) is important to the function of ant repellents. In honeybees and wasps, the role of n-alkanes (a dominated hydrocarbon) was well studied in the field of nest mate and egg recognition (Martin and Drijifhout, 2009).

A study based on the proboscis extension reflex (PER), in honeybees showed that honeybees were unable to discriminate between n-alkanes, whereas they could learn and discriminate between alkenes. When the n-alkanes composition in hydrocarbon profile was altered, it had little effect on protection from 'guard' bees. In contrast, when the n- alkenes (the minor peaks of hydrocarbon profile) composition was altered, honeybees were attacked more intensively by 'guard' bees. Thus, n-alkenes even though present in the small quantity, gives protection against 'guard' bees. It is reported that different colonies of honeybees significantly differ in their hydrocarbon profiles (Machovic *et al.*, 2017). There are three major types of hydrocarbons present in all insects including alkanes, alkenes and methyl hydrocarbons. According to previous FTIR studies of insect wings, the proportion of chemical components of wing was found to be similar among different species (Gibbs and Pomonis, 1995; Ibitoye *et al.,* 2018).

The second predominant compounds present in the butterfly wing region include alkyl halides, alcohols and phenols. These compounds act as a signalling molecule which is useful for insect communication. Understanding the concept of chemical signalling and communication is of vital importance because it is responsible for fundamental processes like species and gender recognition (Schlick- Steiner *et al.*, 2006). In *C. nerissa*, the spectra band at 630 cm^{-1} belong to alkyl halides. Alkyl halides are compounds that carbon group banded with halogen. In *C. pyranthe*, the spectra band at 597 cm^{-1} represent alkyl halides. In *P. ceylanica,* the band at 1539cm^{-1} was assigned to be carbonyl group. These are characterized as C=O stretch. The spectral bands at 649, 560 and 830 cm^{-1} correspond with alkyl halides. In *A. lyncida*, the spectra band

Table 1. FTIR Spectra (Hydrocarbons and Functional groups) of Pierid Butterflies

Hydrocarbons and Functional groups	Butterfly species with its spectral peak values (cm^{-1})								
	Cepora nerissa	*Catopsilia pyranthe*	*Pareronia valeria*	*Pareronia ceylanica*	*Colotis danae*	*Eurema hecabe*	*Appiasly ncida*	*Colotis etrida*	*Ixias pyrene*
Alcohols and Phenols	3667	3687	3703, 3435	3287	3286	3256		3704	
Aldehydes							1647	2936	
Aliphatic amine	1234,1164, 1112	1160,1092	1153,1075, 1013	1257,1073, 1017	1049	1077	1237, 1066	1187	1272, 1083
Alkanes	1339,2967, 2915,2836, 1374,1339	1442,1365	2959,2847, 1464,1377	2966,2901, 1436,1351	2953, 2919	2949, 2924, 2861, 1365	2919, 1370	1382	2970, 2919, 2842, 1374
Alkenes	1645	1800, 1647			1655				
Alkyl halides	630	597	626	649,560,830	630	893, 587	850, 623	674, 597	614
Alkynes			3133					3363, 3235	3227
Aromatic amines		1279, 1160				1269		1272	
Aromatic groups		3064, 3004, 2962	893				1433		1433, 896
Bromine	690	690	690	649, 560	690	690	623	674, 597	690
Carbonyl Groups				1539					
Carboxylic acid	3273			1625		1654,1532, 1444	1134	1741, 1678	
Chlorine				830		850	850	830	
Ketones							1647		
Nitrocompound	1444	1545	1326		1533		1527		1527
Primary, Secondary amines		3286	3314						

at 630 cm^{-1} represent alkyl halides. This methyl branched hydrocarbons among insects functions as a sex pheromones and also acts as a kairomones i.e. a signal molecule that is advantageous to the receiver but not to the donor. These physiologically important hydrocarbon molecules also function as an anti-aphrodisiac agent (Gibbs and Pomonis, 1995; Chung and Carroll, 2015). The bands belonging to the asymmetric and symmetric stretching vibrations of aliphatic C-H bonds of methyl, methylene and methane groups can be found in the wave number region between 3000-2700 cm^{-1}. The bands of bending vibrations of CH_2 and CH_3 can be found at about 1450 and 1370 cm^{-1}.

The absorbance spectral peak value of alcohols and phenols are characterized as O-H stretch. In *C. nerissa*, the absorbance peak at 3667 cm^{-1} represent alcohols and phenols. These are characterized as O-H stretch. In *C. pyranthe,* the observed band at 3687 cm^{-1} was assigned to be alcohol and phenols. In *P. valeria*, the peaks at 3703 and 3435cm^{-1} correspond with alcohols and phenols. In *P. ceylanica*, absorbance band at 3287cm^{-1} represent alcohols and phenols. In *C. danae*, the peak at 3286 cm^{-1} correspond with alcohols and phenols. In *E. hecabe*, the spectra band at 3256 cm^{-1} was assigned to be alcohols and phenols. In *C. etrida*, the absorbance band at 3704 cm^{-1} represent alcohols and phenols. Alcohols and phenol were absent in *I. pyrene*. In addition to communication and signalling, these components are involved in nest-mate recognition, task-specific cues, dominance and fertility cues, chemical mimicry,

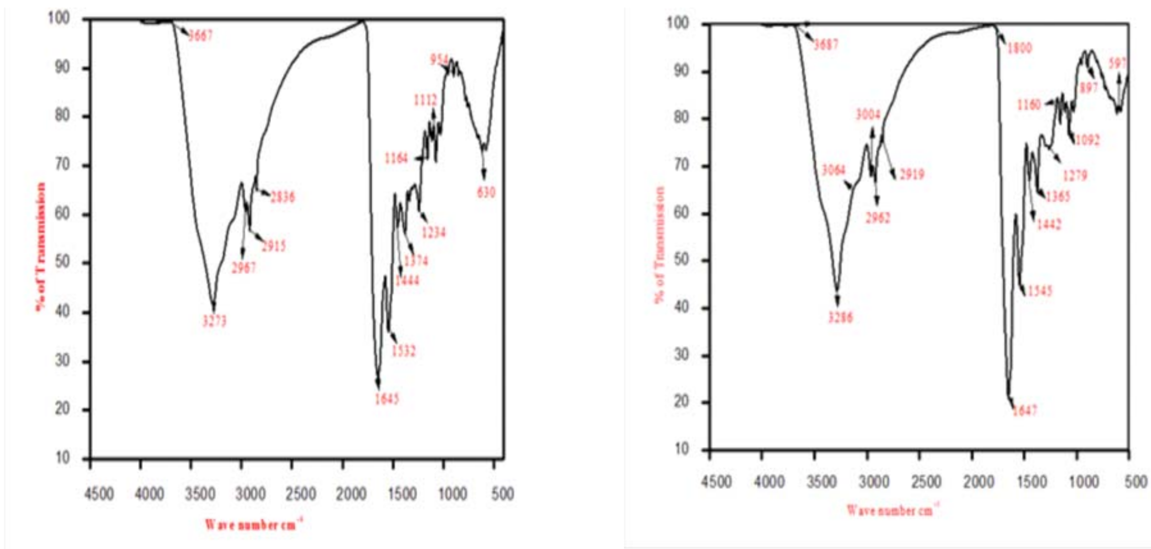

Fig. 1 FTIR analysis of *Cepora nerissa* and*Catopsillia pyranthe*

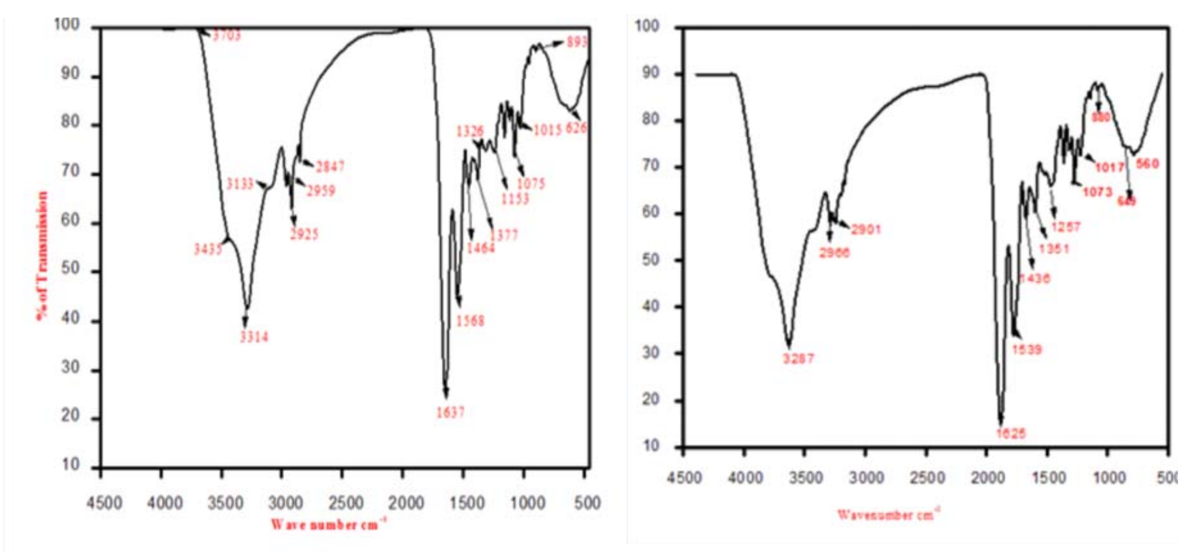

Fig. 2 FTIR analysis of *Pareronia valeria* and *Pareronia ceylanica*

mate selection and kin recognition. All these help to drive the evolutionary force in terms of speciation in butterflies (Rundel *et al.*, 2005).

Insects are smaller in size and have large surface area to the volume ratio, hydrocarbons in the cuticle gives protection against desiccation, by controlling the transcuticular water flux (Martin *et al*., 2004). Hydrocarbon molecules also prevent the wetting of insect's body. Hydrophobic property of hydrocarbons acts against desiccation. Carboxylic acids in the cuticle mainly include amino acids and fatty acids. In *C. nerissa*, the band at 3273 cm^{-1} was assigned to carboxylic acid. These have O-H stretch and carboxylic acids show a strong wideband for the O-H stretch. In *E. hecabe*, the bands at 1654, 1532 and 1444 cm^{-1} represent carboxylic acid. These have C-O stretch of

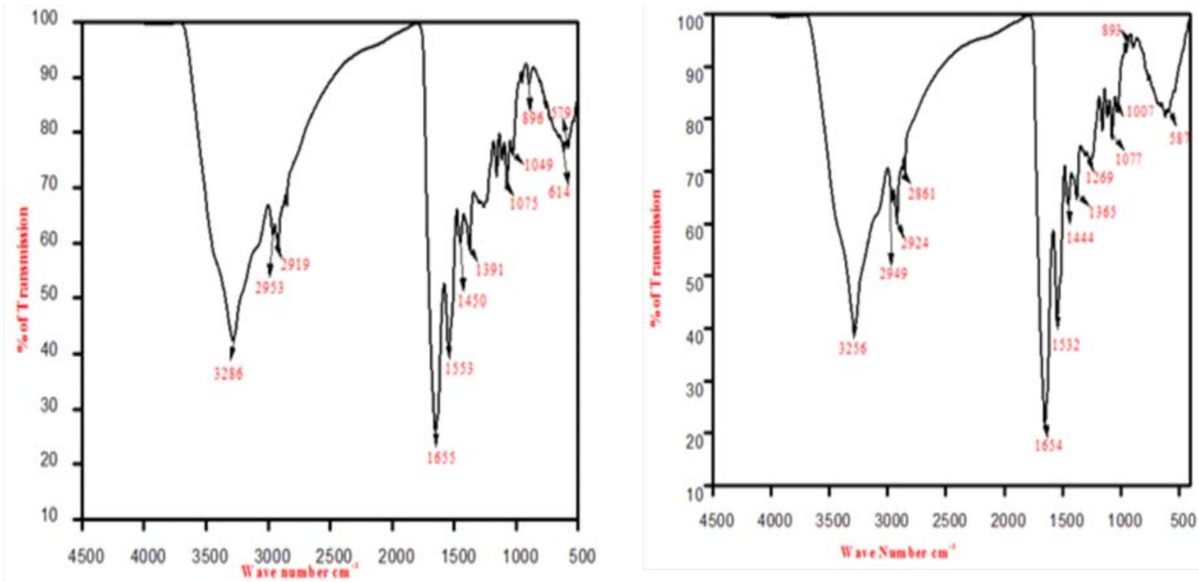

Fig. 3 FTIR analysis of *Colotis danae* and *Eurema brigitta*

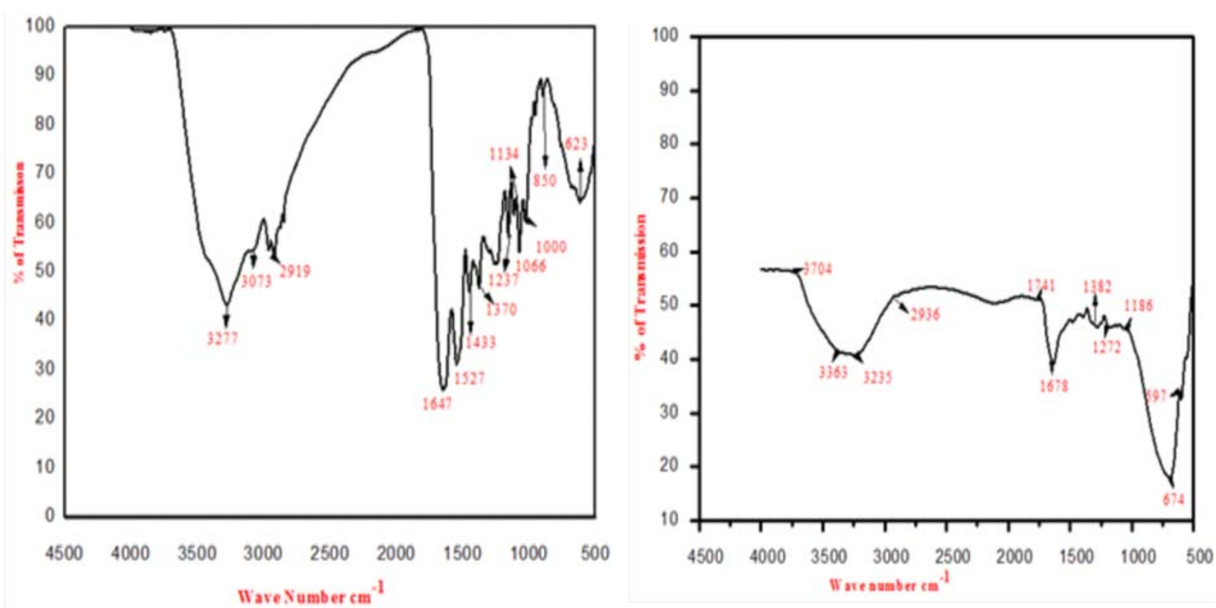

Fig. 4 FTIR analysis of *Appias lyncida* and *Colotis etrida*

carboxylic acids shows a strong wide band. In *A. lyncida*, the spectra bands at 1134 cm^{-1} belong to carboxylic acids. In *C. etrida*, the bands at 1741and 1678 cm^{-1} were assigned as carboxylic Acids. Carboxylic Acids show C=O stretch. The Carboxylic acids present in the insect's cuticle plays an important role in saving the life of insects by acting as an anti-desiccation agent and water proofing agent (Chung and Carroll, 2015). Pieridae butterflies have a specific pigment called pterin (Shamim *et al*., 2014). All pterin pigments have the same basic structure; they differ based on the functional radicals attached to the nucleus. Not all pterin pigments are coloured, colouration depends upon their chemical structure. Majority of butterflies with brown and black scales have melanin, a

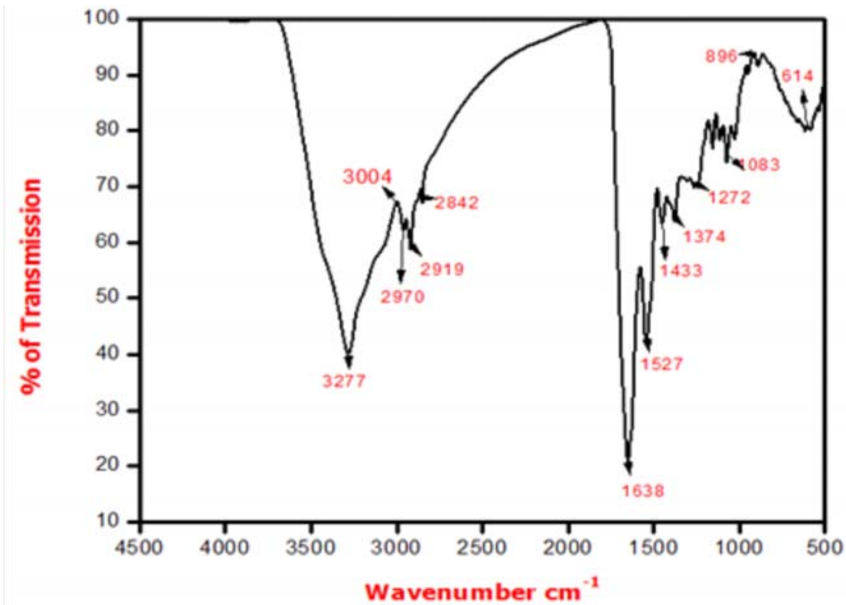

Fig. 5 FTIR analysis of *Ixias pyrene*

universal pigment (Ghiradella, 1998). Pigment melanin contains an essential amino acid phenylalanine, a key compound responsible for wing pigmentation. Insect's wings predominantly have eumelanin rather than pheomelanin. In addition to pigmentation, phenylalanine also has physiological importance, it helps the insects to cope up with parasitic infections (Stuhr *et al*., 2018). The band at 3087cm^{-1} was assigned to be NH stretching modes with some contributions of the aromatic CH stretching vibrations. The protein repeated units give rise to nine characteristic infrared absorption bands namely Amide A, B and I-VII (Tian *et al.*, 2015).

In *C. nerissa*, the absorbance band at 1444 cm^{-1} correspond with N-O asymmetric stretch and the observed absorbance spectra band at 3286 cm^{-1} represent primary and secondary amines and amides. These are characterized as N-H stretching frequency in primary amines and amides are derived from ammonia. Secondary amines and amides show only one peak in the infrared. The observed band at 1545 cm^{-1} was nitro compounds with N-O asymmetric stretch. In *P. valeria*, absorbance peak at 1326 cm^{-1} represent nitro compounds with N-O symmetric stretch and 1568 cm^{-1} was characterized as N-O asymmetric stretch. Band at 3314 cm^{-1} correspond primary and secondary amines and amides. In *C. danae*, the observed peak at 1533 cm^{-1} belong to nitro compounds with N-O asymmetric stretch. In *A. lyncida*, the peak at 1527 cm^{-1} correspond to nitro compounds with N-O asymmetric stretch. In *I. pyrene*, the band at 1527 cm^{-1} belong to nitro compounds with N-O asymmetric stretch.

In *C. nerissa*, the bending of aliphatic amines with C-N stretch and O-H bend was produced at 1234, 1164 and 1112 cm^{-1}. In *C. pyranthe*, the absorbance peak at 1160 and 1092 cm^{-1} represent aliphatic amines. In *P. valeria*, the bands at 1153, 1075 and 1013 cm^{-1} belong to aliphatic amines with C-N stretch and O-H bend. In *P. ceylanica*, the observed bands at 1257, 1073 and 1017 cm^{-1} correspond with aliphatic amines with C-N stretch. In *C. danae*, the absorbance spectra band at 1049 cm^{-1} was assigned as aliphatic amines with C-N stretch and O-H bend. In *A.lyncida*, the observed absorbance bands at 1237 and 1066 cm^{-1} represent aliphatic amines with C-N stretch.

Catecholamine is a precursor of melanin pigment derived from aromatic amino acids such as tyrosine and phenylalanine incorporated into cuticle (Stuhr *et al*., 2018). In *C. pyranthe*, the absorbance peaks at 3064, 3004 and 2962 cm^{-1} belong to the aromatic group. The C-H stretch in aromatic group was observed. The observed absorbance bands at 1279, 1160 cm^{-1} represent aromatic amines, characterized by C-N stretch. In *P. valeria*, the observed absorbance peak at 893 cm^{-1} belong to the aromatic group. In *E. hecabe*, the peak at 1269 cm^{-1} assigned to aromatic amines. The C-N stretch in aromatic amines was observed. *In A. lyncida*, the absorbance peak at 1433 cm^{-1} represent the aromatic group. The C-H stretch in aromatic group was observed. In *C. etrida*, the peak at 1272 cm^{-1} assigned to aromatic amines. This is characterized as a C-N stretch. In *I. pyrene*, the absorbance peaks at 1433 and 896 cm^{-1} were assigned to the aromatic groups. The C-H stretch and C-C stretch in aromatic group were observed.

In all the selected nine butterflies, C-Br stretch (690 - 515 cm^{-1}), was confirmed as bromine. In *P. ceylanica*, a peak at 830 cm^{-1} was characterized as C-Cl stretch. The C-Cl (850-550 cm^{-1}) stretch was confirmed as chlorine. In *E. hecabe*, a peak at 587 cm^{-1} was characterized as chlorine. The presence of bromine and chlorine may be influenced by the colour of the wings. In *A. lyncida* the observed absorbance spectra band at 1647 cm^{-1} correspond with unsaturated aldehydes and ketones. These are characterized as C=O stretch. As in ketones, if the carbons adjacent to aldehyde groups are unsaturated and this vibration is shifted in lower wave number. Spectral peak at 850 cm^{-1} was characterized as C-Cl stretch. In *C. etrida*, the observed absorbance spectra band at 2936 cm^{-1} represent aldehyde. This is characterized as H-C=O; C-H stretch. The spectral peak values at 674 and 597 cm^{-1} were characterized as C-Br. The peak at 830cm^{-1} was characterized as a C-Cl stretch. The C-Cl stretch was confirmed as chlorine. Elements like bromine, sulphur and silica were predominant in wings X-ray fluorescence spectrometry (XRF) analysis of wings revealed the presence of high concentration of Cl, Br and Ca in the supporting structures of wings (Stuhr *et al*., 2018; Kaspar *et al*., 2019).

Cuticular hydrocarbons are also used as a taxonomic tool. These hydrocarbons among insects, they exhibit natural variation and that too under direct selection. During allopartic speciation, these hydrocarbons rapidly diverge due to differences in diet, environmental conditions and this could lead to reproductive isolation (Buckley *et al.,* 2012). Thus cuticular hydrocarbons are better indicator for recent speciation and reproductive isolation (Simmons and Thomas, 2004). Identification of cryptic species is a common problem in taxonomy. Even morphologically indistinguishable species can be discriminated by their hydrocarbon profiles (Schlick-Steiner *et al*., 2006). Long term stability of hydrocarbons allows analyzing the museum specimens and even an extinct species (Martin *et al.*, 2009).

FTIR analysis of nine different Pierid butterfly wings showed many relative sizes of peaks. There are no significant differences in the chemical composition of wings but the colour differences observed among the nine different Pierid butterflies and it was inferred that not majorly due to the differences in the cuticular hydrocarbons. It may be due to the presence of different microstructures like scales, ridges and grooves.

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