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FT-IR, FT-Raman and GC-MS analyses of biochemical compounds in *Ophrys apifera* Huds (Orchidaceae) species

Basel Saleh

Department of Molecular Biology and Biotechnology, Atomic Energy Commission of Syria (AECS), P.O. Box 6091, Damascus, Syria

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Abstract

Fourier-transform infrared spectroscopy (FT-IR), fourier transform raman spectroscopy (FT-Raman) and gas chromatography-mass spectrometry (GC-MS) techniques were employed for biochemical characterization of acetonic, methanolic and ethanolic flowers of *Ophrys apifera* Huds species extracts, as a new *Ophrys* species in Syria. FT-IR spectra 1100 cm^{-1} peak assigned to C–O secondary alcohol stretch C–O stretch (Ethers) and 3000 cm^{-1} peak assigned =C-H stretch (aromatics) groups were mainly detected as a common peaks with the three examined extracts. As for FT-Raman spectra, 1500 cm^{-1} peak assigned to C=C stretch aromatic (aromatics) group was mainly detected as a common peak with the three examined extracts. Whereas, in GC-MS assay, 9-Octadecenamide (Z)- compound (oleamide) was mainly presented as a major and common compound 67.76%, 85.87% and 87.49% with acetonic, methanolic and ethanolic extracts, respectively. All the above mentioned components exhibited a potential role in pharmaceutical and medicine researches and applications. Thereby, more attention should be given to these components to be handled in the future work.

Key words: *Ophrys apifera*, Fourier-transform infrared spectroscopy (FT-IR), fourier transform raman spectroscopy (FT-Raman), gas chromatography-mass spectrometry (GC-MS)

Introduction

Ophrys apifera Hudson is native to Europe (Plants of the World *online*) and belongs to Orchidaceae family includes approximately 35000 species, distributed among more than 1000 genera and 100000 hybrids (Vendrame et al. 2014). Previously, Stewart and Griffiths (1995) reported 800 genera and 25,000 species belonged to Orchidaceae family. This family is one of the biggest families that included approximately 8% to 10% of all flowering plants occurred in the world (Dressler 1981; Pellegrino and Bellusci 2009; Vendrame et al. 2014). Mouterde (1966) reported the occurrence of 11 genera and 45 species belonged to this family in Syria.

To our understanding, the evolution of the Orchidaceae family and genera belonged to it is too hard a cause to their complex flora morphology.

***Corresponding Author**
E.mail: ascientific@aec.org.sy

It has been demonstrated that highly morphological floral diversity within correlated to a high intraspecific variation of some floral characters (Paulus, 2006), leading to complicate the *Ophrys* genus taxonomy where species number belonged to this genus highly variant between 19-> 250 species according to scientists (Delforge, 2005; Pedersen & Faurholdt, 2007).

Moutered (1966) reported the occurrence of 12 *Ophrys* sp. of which *O. apifera* was distributed in Europe, North of Africa and Asia occidental (Lebanon and not in Syria). Whereas, Zohary (1986) reported the occurrence of 8 *Ophrys* sp. in Palestinian flora of which *O. apifera* was distributed in Euro-Siberian and Mediterranean regions.

Too earlier, *O. apifera* was firstly described by Hudson from England in 1762 and that its name refers to the flowers similarity to a bee. Based on this observation, this species known for long time as Bee Orchid as a common name (**http://www.orchidsofbritainandeuropetest.uk/Ophrys%20apifera.html).**

Beside natural beauty of Orchid flowers as ornamental potential, they exhibited multiuse as a food (vanilla), medicine and industrial applications (Zanenga-Godoy and Costa 2003; Vendrame et al. 2014). Orchidaceae displayed a great medicinal importance; their application is too old since 2800 BC as a herbal remedies source by Chines (Chauhan and Chauhan 2014). Their uses as herbal drugs increased worldwide day by day with advanced scientific research.

Some orchids species rich in alkaloids, triterpenoids, flavonoids and stilbenoids (Singh and Duggal 2009) and displayed an antimicrobial activity (Khasin and Rao 1999; Ramesh and Renganathan 2016), anti-inflammatory and antitumor activity (Ramesh and Renganathan 2016) and more recently as antibacterial agent (Sarkar et al. 2018).

Ophrys genus taxonomy based on floral traits morphology has been reported in many investigations (Delforge, 2005; Paulus, 2006; Pedersen & Faurholdt, 2007; Francisco et al. 2015).

Among the listed 12 *Ophrys* sp species according to Moutered (1966) and 8 *Ophrys* sp species according to Zohary (1986) of medicinal plants with conservation status with the highest conservation value, *O. apifera* did not recorded in Syria. In fact, the number of recognized *Ophrys* sp. is steadily increasing due to advanced research and many of them may be encountered in the Syria.

Vibrational spectroscopy based on IR and Raman techniques displayed many advantageous in detecting various elements in biomass, pharmacological and food products *e.g.* presence of known compounds (finger printing) or/and unknown once (functional groups), structural compounds and their alteration, bonds properties (bonds strength and force constants) and phase transitions order parameters and its state.

Resonance fourier transform and Raman spectroscopies are nondestructive analytical methods with less time consuming, don't required special preparation and cheaper than the present 'gold standard' once. They are potential tools for selectively studying chromophores in biological systems (McCann et al. 1992; Urban 1993; Schrader et al., 1999; Yu 2004; Gorzsás and Sundberg 2014).

Many analytical methods are available and successfully employed as a preliminary qualitative analysis of the chemical functional groups occur on the cell wall of biomass (Fox and Whitesell, 1997). Among them, FT-IR and FT-Raman spectra have been employed for biochemical characterization of many biological systems *e.g.* in flax, jute, ramie, cotton, kapok, sisal and coconut fiber (Edwards et al., 1997), milk and ethanolic extracts of poppy (*Papaver somniferum* L.) (Schulz et al., 2004), *Pleuortus ostreatus* extracts (Moharram et al., 2008), seashells of the *Philippine venus* species and sea coral of *Porites* sp (Zakaria et al. 2009), Aqueous leaf extracts of orchid *Rhynchostylis retusa* (Jyoti et al., 2013), *Arabidopsis* (Gorzsás and Sundberg 2014), *Nephelium Lappaceum* leaf extracts (Khan et al., 2015), ethanolic orchid *Geodorum densiflorum* (Lam.) Schltr Pseudobulb extract (Theng and Korpenwar 2015), *Phyllanthus niruri* leaf extracts (Chidambaramc and Janeena 2016), methanolic whole plant orchid *Malaxis rheedei* SW extract (Renjini et al., 2016) and methanolic aerial parts extract of *Artemisia annua* (Hameed et al. 2016). Recently, Barsberg et al. (2018) reported C-lignin with G/S-lignin and lipids in seed coats of three orchid species (*Neuwiedia*

veratrifolia, *Cypripedium formosanum* and *Phalaenopsis aphrodite*) using ATR-FT-IR analysis.

Moreover, many other analytical methods can be used to evaluate and screen primary metabolites in plants. Gas chromatography coupled to mass spectrometry (GC-MS) among them is considered as a common method for this purpose (Osorio et al., 2012); *e.g.* in *Polygonum chinense* L. (Ezhilan and Neelamegam 2012), *Nervilia aragoana* orchid (Aneesh et al., 2013), orchid *Vanda tricolor* (Darmasiwi et al. 2015), *Artemisia annua* (Hameed et al., 2016), *Barleria courtallica* (Sujatha et al., 2017), *Phyllanthus Vasukii* Parthipan (Jemimma et al., 2017), wheat (*Triticum aestivum*) (Lavergne et al., 2018) and more recently in *Hypericum* species (Saleh, 2019).

FT-IR, FT-Raman and GC-MS techniques are advantages for structural, dynamical, thermodynamic and kinetic properties investigation in many biological systems. However, their utility in investigate different vibrational spectra of *Ophrys* genus and in particularly *O. apifera* species is not emphasized in details. Thereby, the current investigation focused on biochemical characterization of *O. apifera* using the above mentioned techniques for this purpose.

Material and Methods

Plant materials and extraction

Ophrys apifera (Orchidaceae) species sampling was carried out at 20 km North Lattakia – Syria and 500 m altitudes above the sea level. Flowers of *O. apifera* were cleaned, shade-dried for 2 weeks, and pulverized to powder in a mechanical grinder. One gram of powder samples was separately extracted in 10 ml of absolute acetone, methanol and ethanol solvents. Tubes were shaken for 5 h and then kept aside and again shaken overnight. Extraction has been continued during 48 h. Filtration with filter papers (Whatman no.1) followed by centrifugation at 1400 g/ 2 min have been done. Solvent from each extract was evaporated using a rotary evaporator under reduced pressure at 40 °C. All dried extracts were then kept in tightly fitting stopper bottles and stored in 4 °C. The final obtained extracts were then subjected to FT-IR, FT-Raman and GC-MS analyses.

FT-IR and FT-Raman assay

The final extracts were used as template for FT-IR and FT-Raman analyses in the wavenumber range of 3500-500 cm^{-1}. IR measurement has been performed using NXR FTIR (Thermo, USA) instrument for FT-IR and NXR FT-Raman (Thermo, USA) instrument for FT-Raman analyses.

GC–MS assay

GC Agilent technologies 6890 N network GC system, supported with Agilent technologies 5973 inert Mass Selective Detector (Agilent, USA) has been employed to investigate biochemical components in acetonice, methanolic and ethanolic *O. apifera* flowers extracts. GC–MS analysis has been performed according to the following conditions: The range scan was 50-350 MU, the column [(DB-35-MS (30 m × 0.25 mm × 0.25 mm)], carrier gas (1.2 ml/min flow of Helium gas). Oven temperature was programmed initially at 65 °C for 4 min, then an increase by 3°C /1 min tell 160 °C, (160 °C /1 min) then 166 °C followed by 6 °C /1 min increasing tell 180 °C (180 °C /10 min). Injector temperature was 250°C and detector temperature was 280 °C and ionization energy was 70 ev. Each extract component was identified by comparing retention time values of gas chromatography on polar columns and by comparing mass spectrum and Nist library databases.

Results and Discussion

Spectra analysis of *O. apifera* flowers extract revealed the appearance of 9, 6 and 5 peaks in acetonic, methanolic and ethanolic extracts, respectively with FTIR (Figure1 and Table 1). Of which, two peaks were common among the three examined solvents: 1100 cm^{-1} peak assigned to C–O secondary alcohol stretch C–O stretch (Ethers) and 3000 cm^{-1} peak assigned =C-H stretch (Aromatics) groups in the case of FTIR spectra (Table 1). Whereas, 4, 2 and 4 peaks were detected in acetonic, methanolic and ethanolic extracts, respectively with FT-Raman (Figure 2 and Table 2). Of which, one peak at 1500 cm^{-1} assigned to C=C stretch aromatic (Aromatics) group was mainly detected as a common peak with the three examined extracts in the case of FT-Raman spectra (Table 2).

Whereas, for GC-MS assay, 5, 4 and 3 peaks were detected in acetonic, methanolic and ethanolic

extracts, respectively (Table 3). Of which, 9-Octadecenamide (Z)- compound (Oleamide) was detected as a common peak with the three examined extracts (Table 3).

Data presented in Table 3 revealed that the possible constituents recorded from acetonic extracts, were 9-Octadecenamide (Z)- (67.76%), followed by Silane, triethyl(pentafluorophenyl)- (18.63%), Hexadecanamide (2.73%), Nonahexacontanoic acid (0.72%) and Heptadecane, 2,6,10,15-tetramethyl (0.66%). As for methanolic extracts, 9-Octadecenamide (Z)- (85.87%), Hexadecanamide (3.06%), Phenol, 4-Fluoro (1.41%) and Propenic acid, 2-cyano-3-dimethyl aminomethylenamino-, ethyl ester (0.45%) were detected. Whereas, in ethanolic one, 9-Octadecenamide (Z)- (87.49%) followed by 1,3,5-Triazin-2, 4-diamine, N,N'-diethyl-6-methoxy (9.2%) and Hexadecanamide (1.75%) were detected.

It has demonstrated that the carboxylic acid functional group displays a cardinal role in the biochemistry of biological systems as well as in drugs design worldwide as anti-inflammatory drugs (NSAIDs), antibiotics, anticoagulants, and cholesterol-lowering statins (Ballatore et al., 2013). Whereas, Tiwari et al. (2015) reported phenolic compounds (C–O secondary alcohol stretch C–O stretch) significance in herbals since they act disrupting the bacterium cell wall, interfering with the ATP pool and altering its membrane potential, causing finally bacterium's death. While, Murti et al. (2011) reported that the =C-H stretch aromatic group as triazole acts as isonicotinamide, antimicrobial and anti-inflammatory agents.

Jyoti et al. (2013) reported bioactive compounds in aqueous leaf extracts of orchid *Rhynchostylis retusa* using FT-IR. The previous study revealed that alkynl, carbonyl, amine and alcohol / phenol groups were presented in FTIR spectra. Whereas, Theng and Korpenwar (2015) applied FTIR to determine the bioactive compounds of ethanolic orchid *Geodorum densiflorum* extract. The previous study revealed the presence of 19 peaks corresponding to alcohols, phenols, alkanes, aldehydes, alkenes, carboxylic acids, esters, ethers, aliphatic amines, amides, sulfides and alkyl halides compounds.

Moreover, Renjini et al. (2016) reported chemical compounds in methanolic orchid *Malaxis rheedei* SW whole plant extract using FT-IR assay. The previous study revealed 9 peaks, of which N-H Stretch (amine) functional group was presented as a major compound among identified chemical compounds.

As for GC-MS assay, from the data presented in Table 3, it worth noting that the 9-Octadecenamide (Z)-compound (Oleamide) was mainly presented as a major and common compound of 67.76%, 85.87% and 87.49% with acetonic, methanolic and ethanolic *O. apifera* flower extracts, respectively. Other investigations reported the biological activity of 9-Octadecenamide (Z)- (oleamide) compound as anti-inflammatory and antibacterial activities (Idan et al., 2015), anti-inflammatory and anti-cancer properties (Hameed et al., 2016), antimicrobial and anti-inflammatory (Jemimma et al., 2017; Sujatha et al., 2017) activities.

Recently, Alabi et al. (2018) reviewed oleic acid and its primary amide (oleamide) for their biological activity and reported their important role as antibacterial and antifungal activities.

GC-MS assay has been employed to investigate bioactive compounds in Orchid species. However, its utility does not yet emphasized in *Ophrys* species. In this regards, Aneesh et al. (2013) reported bioactive components of ethanolic, etheric and methanolic of *N. aragoana* rhizomes orchid extracts using GC-MS. The previous study revealed occurrence of 7, 7 and 3 bioactive components using ethanolic, etheric and methanolic extracts, respectively. Whereas, Theng and Korpenwar (2015) applied the same technique to determine the bioactive compounds of ethanolic orchid *G. densiflorum* extract. The previous study revealed the presence of 4 peaks corresponding to pentane, 1, 1-diethoxy- (30.30%), propane, 1, 1, 3-triethoxy (60.91%), neotigogenin (4.38%) and sarsasapogenin (4.38%). Moreover, Darmasiwi et al. (2015) reported bioactive compounds in hexane:acetone (9:1) of orchid *Vanda tricolor* flower extracts using same technique. The previous study revealed that the compounds presented were fatty acid derivates, monoterpenoids, sesquiterpenoids, benzenoids, phenylpropanoids, hydrocarbons and other oxygenated compounds.

This assay has been successfully employed to investigate bioactive compounds in wide spectrum of plant species. In this regards, Ezhilan and Neelamegam (2012) applied GC-MS to describe chemical compounds in ethanolic *Polygonum chinense* L. extract. The previous study revealed that triterpene compound–squalene (47.01%), and a plasticizer compound–1,2-benzenedicarboxylic acid, mono[2-ethylhexyl]ester (40.30%) were mainly presented. Whereas, Hameed et al. (2016) reported the occurrence of 49 peaks of biological compounds in the methanolic flower *Artemisia annua e*xtracts. Indeed, Sujatha et al. (2017) reported occurrence of 23, 25 and 28 bioactive compounds in the ethanolic extracts of root, stem and leaf of *B. courtallica*, respectively.

Recently, Lavergne et al. (2018) applied GC-MS to characterize wax composition in 4 wheat (*Triticum aestivum*) cultivars. The previous study revealed 263 detected and included 58 wax compounds *e.g.*, alkanes and fatty acids. Indeed, primary alcohols *e.g.* 6-methylheptacosan-1-ol and octacosan-1-ol were higher in leaves compared to stems.

Conclusion

In conclusion, phytochemical characterization of *O. apifera* using acetonic, methanolic and ethanolic flower extracts has been performed based on FT-IR, FT-Raman and GC-MS techniques. The current investigation could suggest the importance of *O. apifera* as a medicinal plant due to its richness in secondary metabolic components. Based upon FT-IR and FT-Raman data, make it as an anti-inflammatory drugs (NSAIDs), antibiotics, anticoagulants, and cholesterol-lowering (due to carboxylic acid), anti-herbal and antibacterial agents (due to C–O secondary alcohol stretch C–O stretch phenolic compounds) and as isonicotinamide, antimicrobial and anti-inflammatory agents (due to =C-H stretch Aromatic group). Moreover, presence of 9-Octadecenamide (Z)- (oleamide) compound in GC-MS analysis, as a major and common compound make it as a good candidate as anti-inflammatory, antibacterial, antimicrobial, and anti-cancer properties. The above mentioned compounds need further attention in pharmacology and medicine applications.

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Table 1: FT-IR analysis of *O. apifera* flowers

Solvent	Peak No	IR frequency (cm^{-1})	Observed IR (cm^{-1})	Bond	Functional groups
Acetone	1	600-500	500	C-I stretch	Aliphatic iodo compounds
	2	900-690	900	=C-H loop bend	Aromatics
	3	1200-1000	1100	C–O secondary alcohol stretch C–O stretch	Ethers
	4	1300-1200	1250	C–O stretch	Carboxylic acids
	5	1475-1365	1390	C–H bend	Alkanes
	6	1600-1400	1500	C=C stretch aromatic	Aromatics
	7	1790-1740	1750	C=O stretch	Anbvdrides
	8	3000	3000	=C-H stretch	Aromatics
	9	3095-3075	3075	C–H stretch	Vinyliden
Methanol	1	1000-650	700	C–H bend	Alkanes
	2	1200-1000	1100	C–O secondary alcohol stretch C–O stretch	Ethers
	3	1600-1400	1500	C=C stretch aromatic	Aromatics
	4	2970-2850	2875	C–H stretch	Alkanes
	5	3000	3000	=C-H stretch	Aromatics
	6	3600-3200	3375	O–H stretch	Hydrogen bonded alcohols, phenols
Ethanol	1	1000-650	700	C–H bend	Alkanes
	2	1200-1000	1100	C–O secondary alcohol stretch C–O stretch	Ethers
	3	2970-2850	2875	C–H stretch	Alkanes
	4	3000	3000	=C-H stretch	Aromatics
	5	3600-3200	3375	O–H stretch	Hydrogen bonded alcohols, phenols

Table 2: FT-Raman analysis of *O. apifera* flowers.

Solvent	Peak No	IR frequency (cm^{-1})	Observed IR (cm^{-1})	Bond	Functional groups
Acetone	1	900-690	900	=C-H loop bend	Aromatics
	2	1600-1400	1500	C=C stretch aromatic	Aromatics
	3	1790-1740	1750	C=O stretch	Anbvdrides
	4	3000	3000	=C-H stretch	Aromatics
Methanol	1	1200-1000	1100	C–O secondary alcohol stretch C–O stretch	Ethers
	2	1600-1400	1500	C=C stretch aromatic	Aromatics
Ethanol	1	1200-1000	1100	C–O secondary alcohol stretch C–O stretch	Ethers
	2	1300-1200	1300	C–O stretch	Carboxylic acids
	3	1600-1400	1500	C=C stretch aromatic	Aromatics
	4	3000	3000	=C-H stretch	Aromatics

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Table 3: GC-MS analysis of *O. apifera* flowers.

Solvent	Peak No	RT (min)	Name of Compound	Molecular formula	Molecular weight	Molecular structure	Peak area (%)
	1	48.34	Heptadecane, 2,6,10,15-tetramethyl	$C_{21}H_{44}$	296		0.66
	2	49.61	Hexadecanamide	$C_{16}H_{33}NO$	255		2.73
Acetone	3	51.28	Nonahexacontanoic acid	$C_{69}H_{138}O_2$	998		0.72
	4	55.23	9-Octadecenamide (Z)-	$C_{18}H_{35}NO$	281		67.76
	5	58.29	Silane, triethyl(pentafluorophenyl)-	$C_{12}H_{15}F_5Si$	282		18.63
	1	18.9	Phenol, 4-Fluoro	C_6H_5FO	112		1.41
	2	49.6	Hexadecanamide	$C_{16}H_{33}NO$	255		3.06
Methanol	3	51.66	Propenic acid, 2-cyano-3-dimethyl aminomethylenamino-, ethyl ester	$C_9H_{13}N_3O_2$	195		0.45
	4	55.23	9-Octadecenamide (Z)-	$C_{18}H_{35}NO$	281		85.87
	1	49.63	Hexadecanamide	$C_{16}H_{33}NO$	255		1.75
Ethanol	2	53.94	1,3,5-Triazin-2, 4-diamine, N,N'-diethyl-6-methoxy	$C_8H_{15}N_5O$	197		9.2
	3	55.25	9-Octadecenamide (Z)-	$C_{18}H_{35}NO$	281		87.49

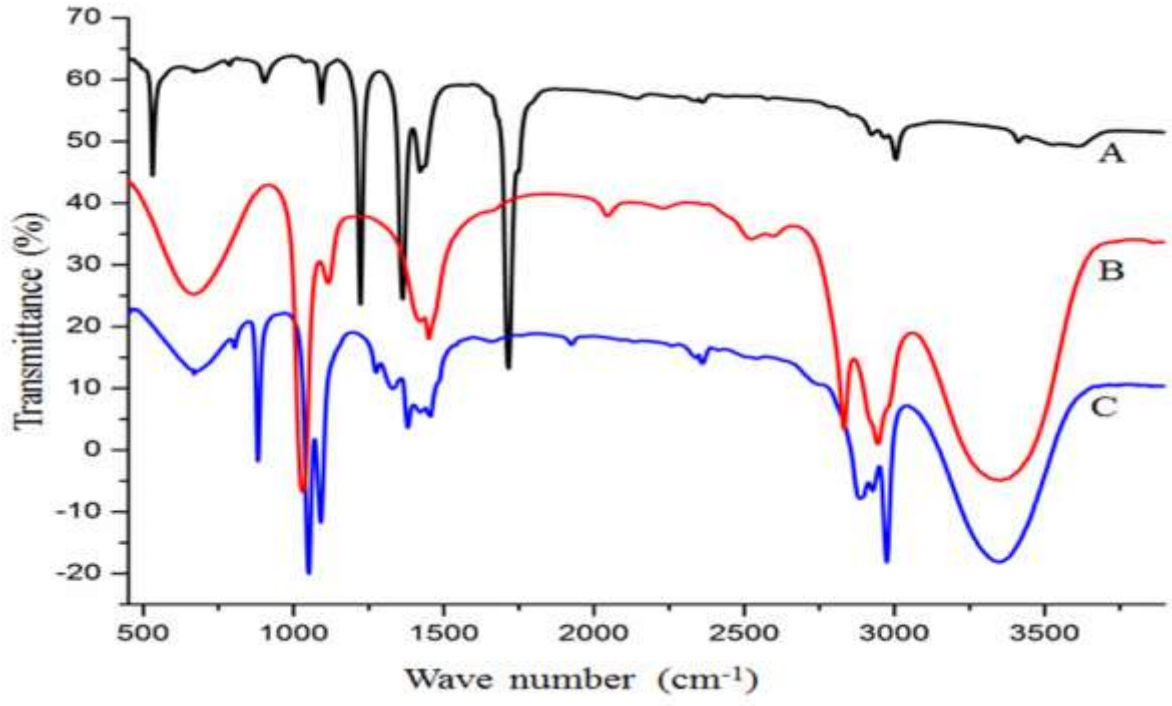

Figure 1. Observed FT-IR vibration wavenumbers of *O. apifera* flowers using A: Acetone, B: Methanol and C: Ethanol solvents.

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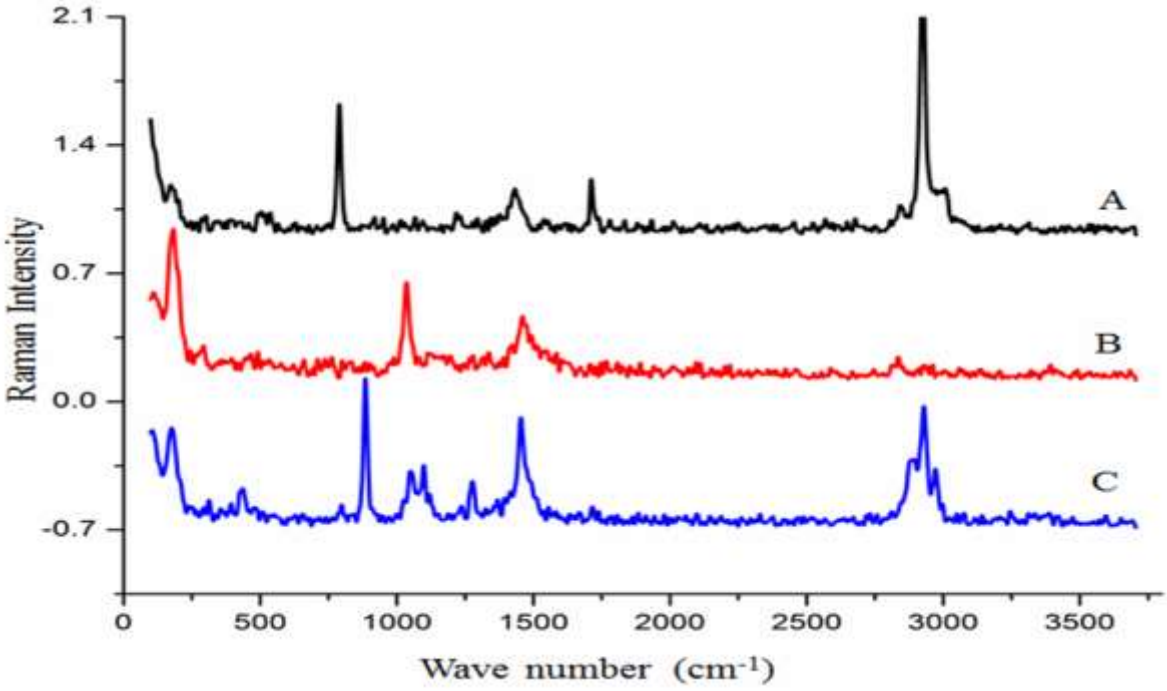

Figure 2: Observed FT-Raman vibration wavenumbers of *O. apifera* flowers using A: Acetone, B: Methanol and C: Ethanol solvents.

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