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Research Article
Analysis of Chemical Composition of Mulberry Silkworm Pupal Oil with Fourier Transform Infrared Spectroscopy (FTIR), Gas Chromatography/Mass Spectrometry (GC/MS) and its Antimicrobial Property

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Abstract

Background and Objective: The antibacterial actions of long-chain unsaturated fatty acids are usually attributed to inhibit many pathogenic microorganisms. The objective of the present study was to analyze the chemical composition of pupal oil of mulberry silkworm with fourier transform infrared spectroscopy (FTIR) and Gas Chromatography/Mass Spectrometry (GC/MS) and to evaluate the isolates for possible *in vitro* antibacterial activity. **Materials and Methods:** Pupae of the silkworm *Bombyx mori* were produced at Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow District of Uttar Pradesh, India, by conducting silkworm rearing as per protocol. The silkworm pupal oil was extracted by using maceration method. Further silkworm pupal oil was analyzed with Model name as Nicolet 6700 Trade Mark Spectrometer and GC/MS. The antimicrobial activity was test by Minimum Inhibitory Concentration (MIC) method using Fuente method. The data was analyzed by One-way analysis of variance (ANOVA) using SPSS program. **Results:** The FTIR analysis of pupal oil proved the presence of alkenes, alkanes, alkynes, organic halogen compounds, aromatic compounds, ethers, esters, aldehydes, alcohols, carboxylic acid, amides and phenols and amides. The GC/MS analysis of pupal oil revealed the existence of the Stearic acid, Palmitic acid and Linoleic acid. **Conclusion:** The FTIR and GC/MS analysis of pupal oil proved the presence of different useful compounds.

Key words: Antagonistic potentiality, antibacterial activity, extraction, FTIR and GC/MS analysis, mulbery Silkworm pupal oil, *Staphylococcus sciuri*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mulberry Silkworm (*Bombyx mori*) is used as food and medicine in Asian countries. It consumed as food particularly by cardiac and diabetic patients as well as people having bronchial asthma, primary trigeminal neuralgia, facial palsy, pain vocal nodules and polyps[1]. The main ingredients silkworm pupae are reported as protein 51%, essential fatty acids 29%, cholesterol 3%, chitin and vitamin A, B_2 and D, with these vitamins being both safe and very important to the human being[2,3]. It also possesses anti-juvenoid[4] immune booster[5] anti-oxidant[6] and estrogenic effects[7]. Currently, it has been reported that fermented silkworm powder has protective effect in alcohol induced hepatotoxicity in a rat model[8].

Moreover, FTIR analysis of mulberry silkworm pupae powder insinuated the presence of alkanes. The absorption arising from C-H region stretching occurs in region between 3000-2840 cm^{-1}. Bands at 2926.2 and 2925.8 cm^{-1}, occurred in mulberry silkworm pupae before and after extraction of pupal oil represent asymmetrical stretching of CH_2 methylene group present in alkane[9].

The natural lipid of desilked silkworm pupae was considered to be a fine resource of alpha linolenic acid. this is based on copious journal report on fatty acid composition of natural lipid mainly of silkworm pupae, *Bombyx mori*[10,11] even though there were variations in the level of alpha linolenic acid reported so far in the *B. mori* silkworm pupae[12].

Alpha linolenic acid is a member of polyunsaturated fatty acids which widely distributed in animal[13]. Nowadays, alpha linolenic acid is used to prevent a variety of disease such as Cardiovascular[14] hypertension, inflammatory and autoimmune disorders[15]. This is because it can produce eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the body by a series of chain elongation and desaturation[16].

The widespread of overuse and inappropriate use of antibiotics in pharmacy which was inevitably increase the emergence of resistant bacterial strains[17] and the increasing rate of antibiotic resistance that exceeds the pace of the growth of innovative antibiotics[18]. The improvement and implementation of latest antibiotics are necessary. Antimicrobial peptides both artificial and natural form has raise interests as antimicrobial agents[18]. Among the potential candidates for new antimicrobial agents, AMPs deserve special attention[19,20]. Natural AMPs have been isolated from several organisms, ranging from bacteria to advanced eukaryotes[21].

Silkworm pupal oil could be a tremendous lipid source for humans because of the presence of rich amount of omega-3 fatty acid. With the recent accent on increasing ingestion of omega-3 fatty acid, the use of silkworm pupal oil in food processing may be satisfactory. The Food and Drug Administration considers daily omega-3 supplementation of up to 3 g to be "Normally regarded as safe." The Silkworm pupal oil has numerous health benefits. The therapeutic benefits of omega-3 fatty acids, which are plentiful in some fish oils, have long been known, dating back to at least the 1950 s, when cod liver oil was found to be effective in treating ailments like eczema and arthritis. In the 1980 s, scientists reported that Eskimos eating a fish-rich diet enjoyed better coronary health than counterparts consuming mainland foods.

In the present study, Mulberry Silkworm (*Bombyx mori*) pupal oil was extracted and analyzed for its antibacterial property against *Staphylococcus sciuri* strain CD97. Primary investigation of pupal oil was done with FTIR to identify the functional group of unsaturated carbon as alkene and alkyne. Further, the presence of compounds of pupal oil was analyzed with GC/MS technique.

MATERIALS AND METHODS

The experiment was carried out at Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University (A Central University) Raebareli Road, 226025 Lucknow (U.P) in the year 2016.

Preparation of pupae powder: Pupae of the silkworm *Bombyx mori* were produced at Department of Applied Animal Science, Babasaheb Bhimrao Ambedkar University, Lucknow District of Uttar Pradesh, India, by conducting silkworm rearing as per protocol suggested by Krishnaswami *et al.*[22]. The pupae powder was prepared as the protocol suggested by Wijayasinghe and Rajaguru[23]. For sample preparation, the collected pupae were dried in hot air oven at 60°C for about 12 h until the pupae became completely dry and then ground by using mixer grinder to open the chitinous coating and to take out the pupal matter. The chitinous coating was then separated manually from the pupal powder.

Extraction of mulberry silkworm pupal oil: The extraction of mulberry silkworm pupae oil was carried out by following maceration method[24]. To prepare extract two different

solvents were used petroleum and ether respectively. About 100 g of mulberry silkworm pupal powder was added to 150 mL of both the solvents separately in a reagent bottle. The reagent bottle was closed tightly and sealed with glycerin to avoid evaporation. The contents of the reagent bottles were filtered with filter paper, into petri dish after 7 days when the colour of the solvent changed to yellow. The filtrate was kept in the open in shed room to evaporate all volatile solvent in which the oil was extracted . The extracted pupal oil was used for further experimental purpose.

Fourier transform infrared spectroscopy (FTIR) analysis of mulberry silkworm pupal oil: The fourier transforms infrared spectroscopy (FTIR) of mulberry silkworm pupal oil, was analyzed using Nicolet TM spectrometer (Thermo Scientific, USA). Pupal oil samples were kept in sample chamber of FTIR for analysis. Spectra were recorded in the mid-IR region 4000-400 cm^{-1} at resolution 4 cm^{-1} with 16 scans. The interferometer and the detector chamber were purged with dry nitrogen to remove spectral interference due to atmospheric carbon dioxide and water vapor. Air background spectrum was recorded before each sample were performed in triplicates.

GC-MS of mulberry silkworm pupal oil: The methylation of mulberry silkworm pupal oil was done by H_2SO_4-MeOH methylation method[25]. For this the reagent were prepared by dissolving 1g of mulberry silkworm pupal oil into 100 mL of the reagent H_2SO_4/MeOH/Toluene (1:10:20) and refluxed on a water bath for 1 h. The reaction mixture was diluted with 1 mL of distilled water and extracted with hexane. The organic layer was transferred to a vial containing anhydrous sodium sulfate. Evaporation of solvent and the sample is ready for GC-MS analysis.

Assembly and conditioning of the column for GC (gas chromatography): The Methylated fatty acid was analyzed for identification of its contents by Gas Chromatography (GC), using a column Perkin Elmer Auto system XLGC and for GC-MS the column was DB-5 (30 m×0.25 mm×0.25 μm). The oven temperature program of GC-MS was as follows: 70°C (3°/min) 250°C (6°/min) 290-60°C (3°C/min) 220°C (7°C). Helium (He, 1 mL/min was used as a carrier gas, split ratio was 1:90, injecting temperature 250°C in GC-MS and the 290°C in split ratio 1:30, detector at 300°C, hydrogen use as carrier gas 10 Psi in GC.

Determination of minimum inhibitory concentration (MIC): The MIC test was done by using Fuente[26] method. The 5 mL bacterial suspensions was prepared in Muller Hinton Broth (MHB) and to detected growth of bacterial strains *Staphylococcus sciuri* strain CD97 and 1 mL containing bacteria in growth log phase (O.D. 610 = 0.001). The tests were conducted in six test tubes with control, media+bacteria and antibiotic streptomycin at different concentration of 12.5, 25, 50 and 100 μL, respectively. Another six test tubes were taken as media, media+bacteria and oil of mulberry silkworm pupal oil at different concentration of 12.5, 25, 50 and 100 μL, respectively. All the test tubes were incubated at 37°C for 72 h. After incubation, OD (optical density) was taken at 610 nm by spectrophotometer.

Minimum Inhibitory Concentration (MIC) was defined as the lower concentration of which no bacteria was detected on plate. This antibacterial test allows determining the percentage of growth inhibition of the bacteria in presence of different concentration of mulberry silkworm pupal oil.

$$\text{Percentage of growth inhibition}^{27} = \frac{\text{OD of test}}{\text{OD of control}} \times 100$$

Statistical analysis: The experiment was carried in triplicate and the results are expressed as Mean±SD. The data was subjected to One-way analysis of variance (ANOVA) using SPSS program version 21[28]. The $p<0.05$ was regarded as significant.

RESULTS

FTIR and GC-MS analysis of mulberry silkworm pupa oil: The region between 3100-3000 cm^{-1}, showed the presence of C = C-H str. m which peak value is 3009.5 (Fig. 1 and Table 1). This showed unsaturation due to carbon-carbon double bonds, which is related with unsaturated fatty acids presented in mulberry silkworm pupal oil after GC/MS study. It is reported that more number of double bonds showed higher unsaturation and this indicate unsaturated compounds are highly reactive. This is also reported that PUFA (polyunsaturated fatty acid) is very essential fatty acid for human being. The GC/MS results also indicating the presence of alpha linolenic compound (omega-3 fatty acid) which unsaturated fatty acid.

The fatty acid compositions of mulberry silkworm pupal oil (Fig. 2 and Table 2) were stearic acid, linolenic acid, palmitic

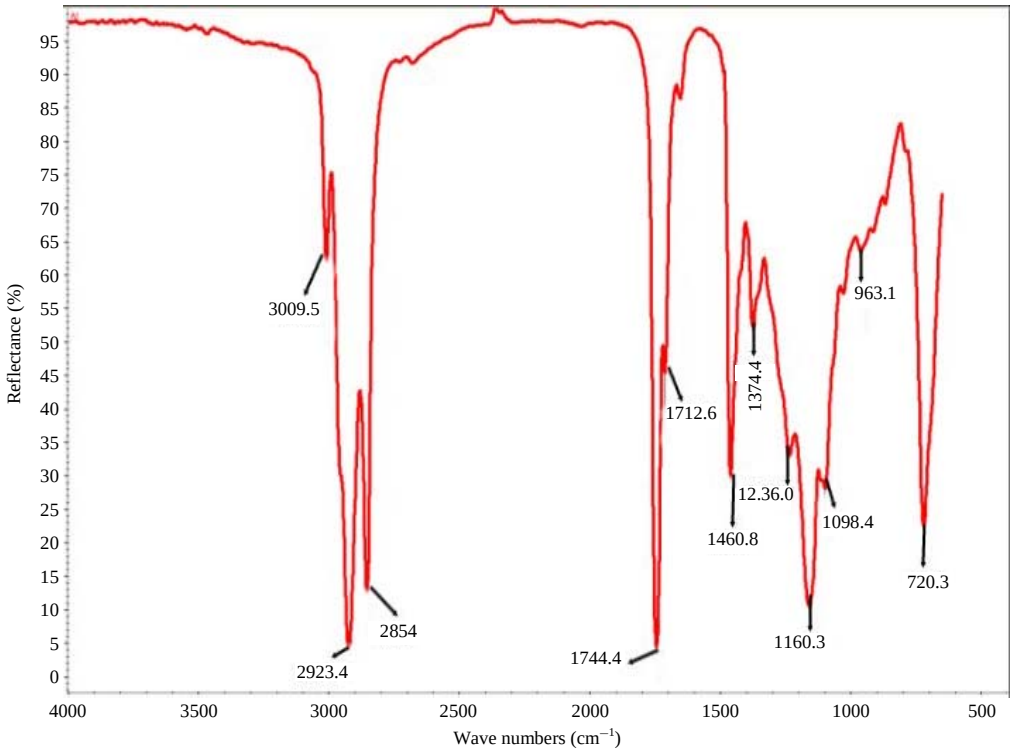

Fig. 1: FT IR spectrum of Mulberry silkworm pupal oil

Table 1: Characteristic absorption frequencies of functional groups for mulberry silkworm pupal oil

Functional groups	Vibration and intensity	Frequency in (cm^{-1})	Peak value
Alkanes	C-H str, m, s	2960-2850	2932.4
			2854.0
	C-H bend, m	1485-1440	1460.8
	C-H bend, m	1470-1430	1460.8
	C-H bend, w	1485-1340	1460.8
			1374.4
			1236.0
	C-C str, w	1300-800	1160.3
			1098.4
			963.1
Alkenes	C=C-H str, m	3100-3000	3009.5
	C-H, m	3040-3010	-
	C-H bend, s	970-960	963.1
	C-H bend, s	915-905	-
Alkynes and Cycloalkanes	C-H str, m	3100-2920	2923.4
			3009.5
Aromatic Compound	Ar-H str, v	3050-3000	3009.5
	C-H bend, s	900-700	720.3
Halogen Compound	C-F str, s	1400-1000	1374.4
			1236.0
			1160.3
			1098.4
	C-Cl str, s	800-600	720.3
Alcohols	O-H str, v, sh	3700-3500	-
	O-H str, v, sh	3570-3450	-
	O-H str, s	3000-2500	2923.4
			2854.0
Phenols	C-O str, s	1400-1310	1374.4
	C-O str, s	1410-1300	1374.4

Table 1: Continue

Functional groups	Vibration and intensity	Frequency in (cm^{-1})	Peak value
Ether	C-O str, s	1270-1200	1236.0
	C-O str, s	1150-1070	1098.4
	C-O str, s	~910	-
Aldehydes	C-H str, w	2900-2820	2854.0
Ketones	C = O str, s	~1745	1744.4
	C = O str, s	1725-1710	1712.6
	C = O str, s	1725-1700	1712.6
	C = O str, s	1715-1690	1712.6
Esters	C=O str, s	1750-1735	1744.4
	C = O str, s	1730-1715	-
Lactones	C = O str, s	1760-1740	1744.4
	C = O str, s	1750-1735	1744.4
Saturated aliphatic acids	C = O str, s	1725-1700	1712.6
	C = O str, s	1715-1694	1712.6
Carboxylic acid	O-H str, w, b	3000-2500	2923.4
			2854.0
Acid anhydrides	C = O str, s	1790-1740	1744.4
	C = O str, s	1770-1725	1744.4
Lactams	C = O str, s	1760-1730	1744.4
Amines	N-H str, m	3500-3300	-
Nitro compound	N = O str, s	1375-1275	1374.4

Table 2: GC-MS analysis of mulberry silkworm pupae oil

Compound names	Molecular formulae	Molecular weight (g mol^{-1})	Retention time (in min)	Peak value	Area (uV*sec)	Area (%)
Palmitic acid 16:0	$CH_3(CH_2)_{14}$ COOH	256.42	40.382	40.56	1000.46	0.65
Linoleic 18:2	$CH_3(CH_2)_4$ CH=$CHCH_2$ CH=$CH(CH_2)_7$COOH	280.44	44.200	45.66	698.91	0.46
Linolenic acid 18:3	CH_3CH_2CH=$CHCH_2$ CH=$CH(CH_2)_7$COOH	278.43	47.090	45.91	1059.67	0.69
Stearic acid 18:0	$CH_3(CH_2)_{16}$ COOH	284.47	49.083	46.77	392.46	0.26

Table 3: Minimum inhibitory concentration of mulberry silkworm pupal oil and Streptomycin on *Staphylococcus sciuri* strain CD97 bacteria

Concentration (µL mL^{-1})	Mulberry pupal oil Mean±SD (OD)	Growth inhibition (%) by mulberry pupal oil	Streptomycin Mean±SD (OD)	Growth inhibition (%) by Streptomycin
Control	0.907±0.005	-	0.3110±0.005	-
12.5	0.887±0.002	12.370	0.1390±0.006	273.505
25	0.787±0.0002	23.423	0.0848±0.005	290.721
50	0.705±0.057	32.475	0.0182±0.003	311.874
100	0.714±0.06007	31.447	0.016343±0.005	312.490
110	0.000±0.000	100.000	0.000±0.000	100.000

acid and linoleic acid. The retention time and peak value are shown in Table 2. The retention time and peak value were high in stearic acid (49.083) and (46.77) and linolenic acid (omega-3 fatty acid) (47.090) and (45.91), respectively (Fig. 2).

Minimum inhibitory concentration test: Mulberry silkworm pupal oil was analyzed for its antibacterial activity against *Staphylococcus sciuri* strain CD97. The bacterial suspension was prepared in Muller Hinton Broth (MHB) and growth was measured by spectrophotometer at 610 nm after 72 h in terms of optical density.

The optical density of mulberry silkworm pupal oil and streptomycin were decreases by increasing the concentration of pupal oil and antibiotic (Table 3) and the percentage growth inhibition also increases by increasing the concentration of pupal oil and antibiotic (Table 3). The very least value of optical density of mulberry pupal oil was 0.714±0.06007 at 100 µL mL^{-1} in comparison to control 0.907±0.005. The very value of optical density of streptomycin was 0.016343±0.005 at 100 µL mL^{-1} in comparison to control 0.311±0.005. *Bombyx mori* silkworm pupal oil significantly ($p<0.05$) inhibited the growth of *Staphylococcus sciuri* strain CD97 at 110 µL mL^{-1} concentrations (Table. 3). The decreasing optical density showed that growth of bacteria inhibited by pupal oil and antibiotic.

Software version : 6.2.0.0.0.B27
Sample name :
Instrument name : Auto_systemXLGC
Rack/Vial : 0/0
Sample amount : 1.000000
Cycle : 1

Date : 3/25/15 3:30:44 PM
Data acquisition time : 3/3/15 10:44:21 AM
Channel : A
Operator : Manager
Dilution factor : 1.000000

Result file: C:\PenExe\TcWS\Ver6.2.0\Examples\FEB_15\LNMA1_030315_-20150303-120009.rst
Sequence file: C:\PenExe\TcWS\Ver6.2.0\Examples\LNMA1_030315.seq

CIMAP

Peak #	Component name	Time (min)	Area (uV*sec)	Area (%)
1		14.174	619.41	0.40
2		23.733	160.48	0.10
3		28.288	10348.08	6.76
4		30.057	651.47	0.43
5		36.412	774.04	0.51
6		40.382	1000.46	0.65
7		43.593	584.69	0.38
8		44.200	698.91	0.46
9		47.090	1059.69	0.69
10		47.871	74230.21	48.52
11		49.083	392.46	0.26
12		50.206	1556.08	1.02
13		51.319	3830.35	2.50
14		53.611	926.03	0.61
15		53.830	33240.36	21.73
16		54.007	1067.48	0.70
17		54.650	18529.85	12.11
18		55.969	583.49	0.38
19		59.506	1077.12	0.70
20		60.911	1000.84	0.65
21		61.759	200.72	0.13
22		66.875	463.25	0.30
			152995.42	100.00

Missing Component Report
Component Expected Retention (Calibration file)

All component were found

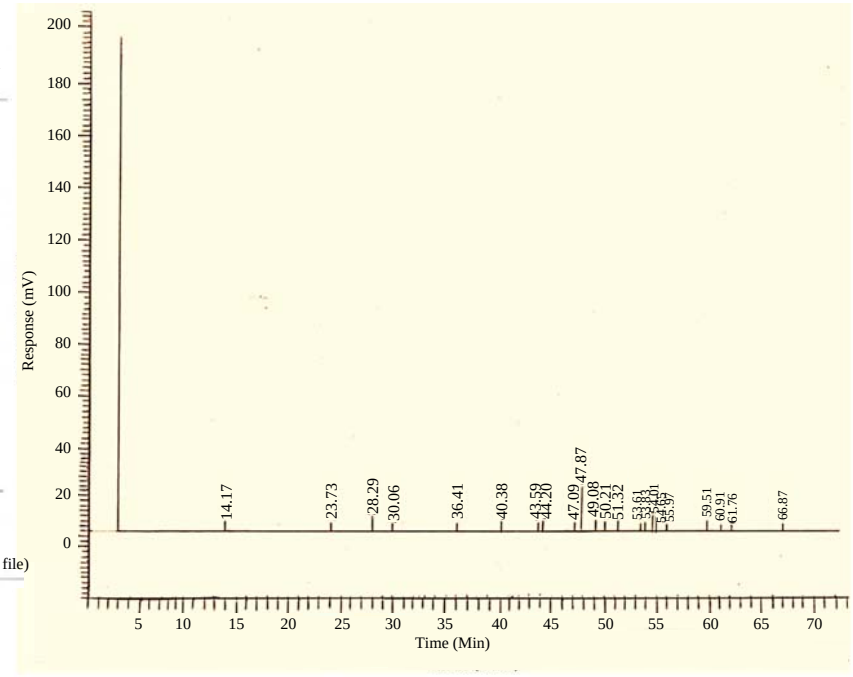

Fig. 2: Retention time of mulberry silkworm pupal oil and GC-MS spectra of mulberry silkworm pupal oil

DISCUSSION

The C=C stretching mode of unconjugated alkenes usually shows moderate to weak absorption between 1667-1640 cm^{-1}. The bands at 1646.2 and 1646.0 cm^{-1} MBSP-B (mulberry silkworm pupae before extracted pupal oil) and MBPB-A (mulberry silkworm pupae after extracted pupal oil) were recorded which indicated C=C stretching in unconjugated alkenes. These bands were not recorded in other samples. The most characteristic vibrational modes of alkenes are the out of plane C-H bending vibration between 1000-650 cm^{-1}. The bands at, 880.7 cm^{-1} in MBSP-B, 880.0 cm^{-1} in MBSP-A may be due to out of plane C-H bending vibration of the vinyl, vinylididene group and trans disubstituted alkenes. Aromatic C-H stretching occur between the region 3100-2900 cm^{-1}. The band occurred at 3011.6, 3010.6, 3012.2, 3012.8, 3011.9, 3013.2, 3012.9 and 2924.8 cm^{-1} in MBSP-A and MBSP-B the samples indicated C-H stretching[9].

Longvah *et al.*[29] investigate mulberry silkworm pupae and found that *B. mori* have16:0 palmitic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid 18:3 alpha linolenic acid, total saturates mono-unsaturates, polyunsaturates 26.2, 7.0, 36.9, 4.2, 25.7, 33.2, 66.8, 36.9 and 29.9%, respectively. In the present study, linoleic acid and alpha linolenic acid were observed in mulberry silkworm pupal oil.

Singh *et al.*[30] studied on Vitellogenin (Vg) from the silkworm, *Bombyx mori*: An efficient anti-bacterial agent. Silkworm, *Bombyx mori*, Vg was isolated from perivisceral fat body of day 3 of pupa. Both Vg sub-units were co-purified as verified by mass spectrometry and immunoblot. Purified Vg responded to specific tests for major post-translational modifications on native gels indicating its nature as lipo-glyco-phosphoprotein. The Vg fraction had strong antibacterial activity against gram-negative bacterium *Escherichia coli* and gram-positive bacterium *Bacillus subtilis*. Microscopic images showed binding of Vg to bacterial cells and their devastation. When infected silkworm larvae were treated with purified Vg they survived the full life cycle in contrast to untreated animals. This result showed that Vg has the ability to inhibit the proliferation of bacteria in the silkworm fluid system without disturbing the regular

metabolism of the host. So, it is indicating that pupae oil also has antimicrobial activity which was shown in present study of mulberry silkworm pupal oil against *Staphylococcus sciuri* strain CD97.

Priyadharshini[31] studied on *in vitro* evaluation of antibacterial activity of chitosan extracted from mulberry silkworm (*Bombyx mori*) pupae against gram-negative (*Escherichia coli*) and gram-positive bacteria (*Bacillus thuringiensis*, *Staphylococcus aureus* and *Enterococcus faecalis*). Different concentrations of chitosan such as 10, 30, 50, 100, 250, 500 and 750 µL were used for this study. Among the different concentrations 750 µL mL^{-1} showed 17.5, 15.0, 11.5 and 14.0 mm of inhibition against *E. faecalis* followed by *E. coli*, *S. aureus* and *B. thuringiensis.* The zone of inhibition was increased with increasing concentrations of chitosan. The antimicrobial activity of chitosan indicated that the pupae generated from silk reeling industries could be used as an effective antimicrobial agent in the pharmaceutical industry. So, during grinding mulberry silkworm pupae some percent of chitosan comes in pupal oil which showed antimicrobial activity against *Staphylococcus sciuri* strain CD97.

CONCLUSION

In present study, an attempt has been made spectral chemical analysis by FTIR, GC-MS and antimicrobial activity of mulberry silkworm pupal oil. The GC-MS analysis of pupal oil revealed the existence of the Palmitic acid, Linoleic acid, Linolenic acid and stearic acid, the identification of chemical compounds was based on the peak area, retention time molecular weight and molecular formula. The MIC test revealed that *Bombyx mori* silkworm pupal oil significantly inhibited the growth of *Staphylococcus sciuri* strain CD97 at 110 µL mL^{-1} concentrations.

SIGNIFICANCE STATEMENT

This present study will help the researchers to uncover of functional groups of Mulberry Silkworm (*Bombyx mori*) pupal oil by FTIR and detect the compounds by GC/MS. The present study will help to know the compounds that are responsible for the antibacterial activity against *Staphylococcus sciuri* strain CD97 present in mulberry silkworm pupal oil.

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