"INVESTIGATIONS ON MULBERRY AND NON MULBERRY SILKWORM PUPAL OIL"

THESIS
SUBMITTED FOR THE A WARD OF DEGREE OF
Doctor of Philosophy
IN
APPLIED ANIMAL SCIENCE

BY
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M.Sc.

UNDER THE SUPERVISION OF
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DEDICATION

I dedicated this study to the loving late mother, **Mrs. Kalawati Devi**, and to my late Father, **Mr. Shikari**, my supervisor **Dr. Venkatesh Kumar R.,** my brother **Mr. Narendra Dev** and my wife **Mrs. Neetu Singh (Sub Inspector U.P. Police)** for their understanding, endless love and care throughout my research work.

DECLARATION

I hereby declare that the thesis entitled "**INVESTIGATIONS ON MULBERRY AND NON MULBERRY SILKWORM PUPAL OIL**" submitted by me under the guidance and supervision of Dr. Venkatesh Kumar R., in partial fulfillment of the degree of Ph.D. in Applied Animal Science in the Department of Applied Animal Sciences, School for Bioscience and Biotechnology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow. It is outcome of my own effort and an original scientific work.

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CERTIFICATE

This is to certify that the thesis titled **"INVESTIGATIONS ON MULBERRY AND NON MULBERRY SILKWORM PUPAL OIL"** submitted by **Mr. Param Dev,** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) regulations -1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Date:

Supervisor

Head of the Department

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ABBREVIATIONS

Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Thr	Threonine
Val	Valine
Trp	Tryptophan
FAO	Food and Agriculture Organization
WHO	World Health Organigation
DHA	Docosahexaenoic acid
GC-MS	Gac chromatography Mass spectrometry
EPA	Eicosapentanoic acid
AMPs	Antimicrobial peptides
MRSA	Methyline Resistance *Staphyllococus aurius*
DNA	Deoxyribonuleic acid
ROS	Reactive oxygen species
SOD	Super oxide dismutase
CAT	Catalase
US	United state
COX	Cyclooxygenase
FFA	Free fatty acid
CTB-APSL fusion protein	CTB-APSL fusion protein
TNF-α	tumor necrosis factor-alpha
IL-6	*Iinterleukin-6*
BmNPV	Bombyx mori nucleopolyhedrovirus
AV	Acid value
KOH	Potassium Hydroxide
I Br	Iodo-bromide
EV	Ester Value
SV	Saponification Value
GLC	Gas Liquid Chromatography
NCBI	National center for Biotechnology Information
H_2SO_4	Sulphuric acid
MeOH	Methnol
PDA	Potato Dextrose Agar
UV	Ultra Violet
PCR	Polymeric Chain Reaction

MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
OD	Optical Density
FRSA	Free radical scavenging activity
DPPH	1-diphenyl 1-2-picrylhydroxyl radical
NBT	Nitro blue Tetrazolium
STZ	Streptozotocin
PUFA	Polyunsaturated fatty acid
TSS	Toxic shock syndrome
SEM	Scanning electron microscopy
HIV	Human Iminuo Virus
Q3MG	3-malynoglucoside
LDL	Density lipoprotein
SOD	Super oxide dismutase
FRAP	Ferric reducing antioxidant power
H_2O_2	Hydrogen peroxide
SOD	Superoxide dismutase
MPE	Methanolic pupae extract
IC_{50}	Inhibitory Concentration at 50%
SFA	Saturated fatty acids
TCA	Trichloroacetic acid
MDA	Malondialdehyde
DM	Diabetes mellitus

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CHAPTER I

GENERAL INTRODUCTION

1. Introduction

Over the last six decades, Indian silk industry has registered an impressive growth, both horizontally and vertically. Plans and schemes implemented by central and state agencies and relentless efforts of thousands of dedicated persons in the fields of research and extension have helped in this context. This sector has the potential to explore the by-products available during different stages of its process. One such area is the utilization of silkworm pupae oils for human benefits. Unfortunately, industries have failed to utilize those resources effectively, particularly silkworm pupae and their oils which are showed huge health benefits.

1.1 Medicinal value of silkworm pupae oils

Pupae oils can effectively reduce triglycerides, prevent and treat fatty livers (**Harris *et al.,* 1997**) protect the liver after consumption of alcohols, improve the blood quality and the environment within the blood vessel, effectively soften the blood vessels, lower blood pressure, and prevent arteriosclerosis and thrombosis. Pupae oils enable the prostaglandins to maintain balance with effects of preventing prostate diseases, improving the functions of insulin-producing beta cells, restoring the fatty acid desaturase activity of cells in diabetic patients and has marked hypoglycemic effect free from reoccurrence (**Gavia *et al.,* 2003**).

1.2 Role of silkworm pupae in food

The human body needs eight kinds of essential amino acids absorbed from food which are Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Valine,

Tryptophan, their contents in silkworms are two times higher than those of pork and four times than those of egg and milk. The silkworm pupae protein is a complete protein and the amino acid compositions are with appropriate proportions in line with FAO/WHO standards **(Xia and Zhao, 2003; Chen *et al.*, 2002a, b)**. Besides, silkworm pupae have abundant fatty acids (mainly unsaturated fatty acid) **(Qian., 1997)**, chitin, vitamins A, D, B_1, B_2, B_5, microelements, antibiosis peptides, hormones, lysozyme. More than 30% of silkworm pupae oils contains linolenic acid, which is the main source of DHA (docosahexaenoic acid) exerting an important effect on human intellect and memory improvement, sight-protection and is a precaution chemical against hyperlipidemia. Moreover, some unsaponifiable ingredients, including b-sterol, cholesterol and campesterol, make up approximately 1% of silkworm pupae fat. Silkworm pupae are an extraordinarily valuable edible animal protein resource.

1.3 Role of omega-3 fatty acids

The alpha-linolenic acid is present in the silkworm pupae oils is used to prevent a variety of diseases such as cardiovascular complications **(Roupas *et al.*, 2006)**, hypertension, inflammatory and autoimmune disorders **(Ferrucci *et al.*, 2006)**, depression and certain disrupted neurological functions **(Christensen *et al.*, 2005)**. This is because it can synthesis eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the body by a series of chain elongation and desaturation **(Trattner *et al.*, 2008)**. Therefore, alpha-linolenic acid is a polyunsaturated fatty acid with prominent bioactivity and is used widely in medicine food and cosmetic industries. The human body cannot synthesis PUFA as linolenic acid (omega-3) and therefore it must be obtained from food. The α-linolenic acid has been found to inhibit the growth of several cancer

cell lines, such as the colon **(Hiroki., 1994)**, larynx **(Alison Colquhoun, 1998)**, and leukemia **(Abbika and Das., 1994)**. Furthermore, it has been suggested that alpha-linolenic acid suppresses as the development of allergies **(Tomio *et. al.*, 1994)**, **(Shiro *et. al.*, 1994)**, obesity **(Masataka *et. al.*, 1997)**, and neuronal death **(Inger *et al.*, 2000)**. Silkworm Pupae oils would therefore be a good source of the functional fatty acids, α-linolenic acid. On the other hand, due to its high degree of unsaturation, alpha-linolenic acid is known to be oxidative less stable and the oxidation of α-linolenic acid in food and nutraceutical.

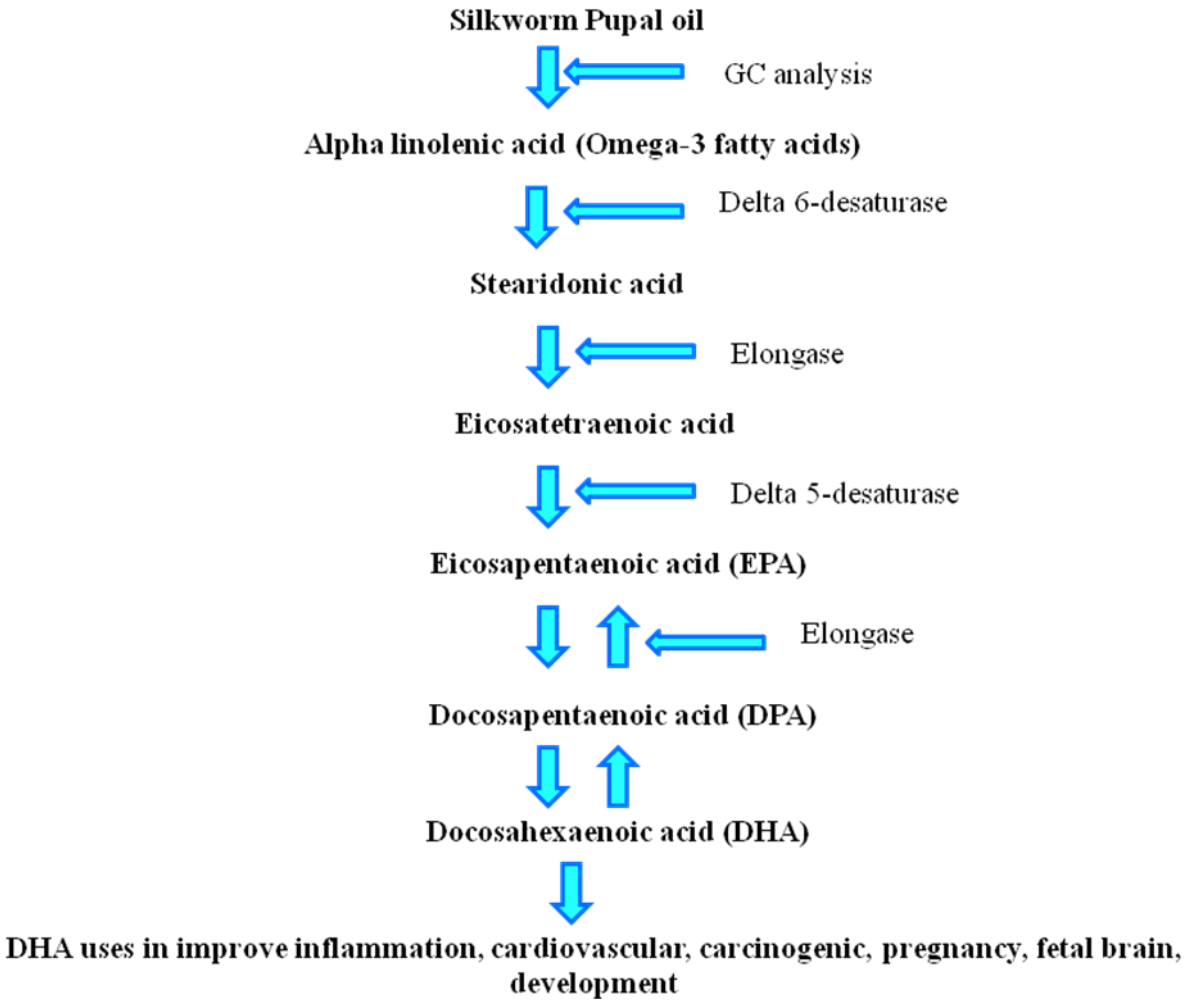


Fig. 1. Role of silkworm pupae oils in medicine

Silkworm pupae oils contain various essential fatty acids with bioactivity and thus can be used as raw material for cosmetics. Silkworm pupae oils extracted by boiling are used in the cosmetic industries for making soap and moisturizers **(Kotake *et al*., 2002)**. The compound present in silkworm pupae oils as palmitic acid and alpha-linolenic acid were the major fatty acids comprising 69–72% of the total fatty acids, which is similar to that reported earlier for eri pupae fats by **(Shanker *et al*., 2006)**.

Several studies revealed that the active substance in silkworm pupae is extracted by the organic solvents are used to determine the antioxidant property, to prevent aging by many oxidants **(Leibovitz and Siegel, 1980; Gutierrez *et al*., 2006)**. The α-linolenic acid content has been reported to be as low as 0.6% in *Heliothis virescens* (commonly known as the tobacco budworm, is a moth of the Noctuidae family found throughout the eastern and southwestern United States) and to as high as 51% in *Hyalophora cecropia* (is North America's largest native moth. It is a member of the Saturniidae family, or giant silk moths) silkworm pupae **(Thompson, 1973)**. There are many reports to show that alpha-linolenic acid in the diet of animals can significantly reduce serum triglyceride levels **(Harris, 1997)**. The aqueous extract of silkworm pupae also reduces serum glucose in diabetic mice, while not affecting normal mice **(Shi *et al*., 1990)**. Silkworm powder can be easily digested and absorbed by human bodies. It also can promote the physiological functions of the gastrointestinal tract. Furthermore, silkworm powder plays an excellent role in lowering blood glucose levels **(Ryu *et al*., 1997)**.

It is becoming apparent that regular consumption of long-chain polyunsaturated omega-3 fatty acids lowers the overall incidence of, as well as the rate of death from, cardiovascular heart disease. The biochemical basis for cardio protective effects of

omega-3 fatty acids are probably multifactorial and may collectively result in increased heart rate variability (anti-arrhythmic), reduced atheroma development (anti-atherogenic), and decreased platelet reactivity/aggregation (anti-thrombotic), which may be mediated by the substrate competition between omega-3 fatty acids and arachidonic acid (C20:4n-6) for cyclooxygenase (COX) enzymes that produce prostaglandins and thromboxane (**Miralaikbari *et al.*, 2006**). In addition, omega-3 fatty acids, namely EPA and DHA, still have other health benefits related to the immune system and renal disorders, as well as inflammation, allergies and cancer. DHA also plays an important role in brain development and retinal function of the fetus and in infants (**Wanasundara et *al.*, 2002**).

1.4 Role of insect in antimicrobial activity

Insects are recognized to have both cellular and humoral immune systems which together form a powerful protection against invading bacteria **(Boman, 1985; Dunn, 1986; Kimbrell, 1991)**. The failure of most important drugs on microorganisms, there is an urgent need to develop and other control agents. *Staphylococcus aureus* is an opportunistic pathogen cause ruthless infections in humans. MRSA (Methicillin-resistant *Staphylococcus aureus*) is a worldwide problem, the number of hospitalized cases increases from last ten years by MRSA. Vancomycin remains the first line intravenous drug for severe MRSA infections (**Deleo *et al.*, 2010, Chambers and Deleo., 2009)**. *Staphylococcus aureus* has a remarkable ability to develop antibiotic resistance, Vancomycin resistance has resulted in a steady decline in the efficacy of these valuable antibiotics (**Ray *et al.*, 2013)**. The resistance to antibiotics is mainly due to the slow growth rate and low metabolic activity of bacteria.

Fatty acids act as a key element of antimicrobial food additives due to their inhibitory stroke on unwanted microorganisms **(Freese, *et al.*, 1973)**. Copious researchers found that the long-chain unsaturated fatty acids such as linoleic and oleic acids are bactericidal to significant pathogenic microorganisms, including methicillin-resistant *Staphylococcus aureus* **(Farrington, *et al.*, 1992; Kabara, *et al.*, 1972; Knapp & Melly, 1986)**, *Helicobacter pylori* **(Hazell & Graham, 1990; Sun, *et al.*, 2003)**, and Mycobacteria **(Seidel & Taylor, 2004)**. The antibacterial actions of long-chain unsaturated fatty acids as oleic acid, linoleic acid are frequently recognized to inhibit against pathogenic microorganisms **(Kabara *et al.*, 1972; Knapp & Melly, 1986; Seidel & Taylor, 2004; Sun *et al.*, 2003)**.

In the *Bombyx mori (B. mori),* cecropins have three subtypes, A, B, and D. It contains two α-helices and they act on gram-negative bacteria most powerfully **(Yamano *et al.*, 1998; Taniai *et al.*, 1995; Yang *et al.*, 1999)**.

1.5 Role of antioxidant

Aging is a widespread procedure and has been defined as the progressive loss of function accompanied by declining fecundity and escalating mortality and disability. Altered response to therapeutic intervention might be measured in any future definitions of aging (**Lean and Counter., 2004)**. Oxidative modification of lipids and small cellular molecules by reactive oxygen species (ROS) along with impaired antioxidant mechanism play some role in an extensive variety of common diseases and age-related degenerative situation. Oxidant injury by ROS is also related to photo aging radiation toxicity, cataract formation and muscular degeneration (**Kolawole *et al.*, 2014)**. Once free radicals are initiated, they can propagate by connecting in chain reactions with other less reactive

types, the resulting chain reaction compounds commonly survive longer in the body and thus amplify the potential for cellular injury. To protect molecules alongside toxic free radicals and other ROS, cells have developed the antioxidant defense system that comprise the enzymes superoxide dismutase (SOD), which dismutase superoxide; catalase (CAT); glutathione reductase and glutathione peroxidase, which destroy toxic peroxides and small molecules including glutathione (**Cheng *et al.*,2014)**. The rise in catalase activity during larval development was directly related to the formation of pro-oxidant in the larva and checks the deleterious effect of aging through its antioxidant effect (**Amritha *et al.*, 2014)**. External sources of antioxidant vitamins like vitamin C, vitamin E and phytochemicals from plant-rich diets provide important protection against oxidant damage (**Borek., 2010)**. The Dietary antioxidant has been demonstrated to be protected through the activation of hermetic pathways, including vitagenes and proteasomal activity degrading oxidatively modified proteins (**Izabela *et al.*, 2014)**.

1.6 Role of diabetes

There are approximately 366 million people worldwide who suffer from diabetes and another 280 million people with pre-diabetes as evidenced by impaired glucose tolerance. In 2011, 4.6 million people died from diabetes, meaning one diabetes-related death for every seven seconds (**International Diabetes Federation., 2011**). Because of its complex disease process, the cure for diabetes is still undiscovered and patients need to receive lifelong treatment. Therefore, the research and development of low toxicity and long-acting diabetes drugs have a very significant impact on the prevention and treatment of diabetes and on improving people's quality of life. Diabetes is mainly divided into type-1 diabetes and type-2 diabetes, wherein more than 90% of all people with diabetes

have type-2 diabetes. The treatment of type 1 diabetes is mainly dependent on exogenous insulin, whereas the treatment of type-2 diabetes often includes biguanides, sulfonylureas, α-glucosidase inhibitors, and other drugs (**Lundgren *et al.*, 2010**).

Studies found that prolonged exposure to high concentrations of free fatty acids (FFA) can cause lipid overload and increased apoptosis of beta cells and reduced insulin secretion (**Lupi *et al.*, 2002, Piro *et al.*, 2002**). The CTB-APSL (cholera toxin B subunit and active peptide from shark liver) fusion protein could obviously improve lipid metabolism and thus has a therapeutic effect on diabetes. A growing number of studies have confirmed that many types of inflammatory factors can predict the occurrence of type-2 diabetes, such as TNF-α (Tumor necrosis factor alpha) and IL-6 (Interleukin 6**)** (**Pickup., 2004, Ortis *et al.*, 2010**). In addition, inflammatory cytokines can cause insulin receptor signal transduction abnormalities, leading to the dysfunction of pancreatic β cells, participation in macrovascular and microvascular complications, and leading to retinopathy, non-alcoholic fatty liver and other serious consequences (**Steer *et al.*, 2006, Vincent *et al.*, 2007**). Meanwhile, clinical trials have confirmed that anti-inflammatory therapy can significantly improve type-2 diabetic patients with abnormal glucose metabolism (**Tsiavou *et al.*, 2004**). In addition, the extracts from silkworm also have anti-diabetic effects (**Ryu *et al.*, 2012**).

1.7 Aim: Investigations of antimicrobial, antioxidant and antidiabetic properties of mulberry and non mulberry silkworm pupae oils

1.8 Objective:

1. To analysis of antibacterial and antifungal properties of silkworm pupae oil of both mulberry and non-mulberry silkworms.
2. To analysis of antioxidant property of pupal oil of mulberry and non-mulberry silkworms.
3. To evaluate the effect of silkworm (mulberry and non-mulberry) pupal oil on glucose uptake.

CHAPTER II

ANTIBACTERIAL AND ANTIFUNGAL PROPERTIES OF MULBERRY AND NON MULBERRY SILKWORM PUPAE OILS

2. Introduction

2.1 Background of the study

Sericulture is one of the high remunerative occupations and still accomplished by villagers for centuries. Silkworm provides food, fiber, and biomedical applications. Normally it is a rich source of fat as well as vitamins and minerals **(Banjo, Lawall, & Songonuga 2006)**. Developing countries like China, India and Brazil used it as their cash crop.

A huge number of waste silkworm pupae produced after the silk reeling and dumping of these waste pupae posses a great threat to the environment. Few researchers have shown that these waste pupae contain 30% lipid **(Sharma and Ganguly 2011)**. Therefore, nowadays these bio-waste dry pupae which are economically important in the field of Pharmacology, Agriculture, as well as the production of biodiesel.

2.2 Omega-3 fatty acids in antimicrobial activity

The antibacterial studies of PUFA extracts from fishes have been very few results to date. Marine lipids, especially n-3 fatty acids such as eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) have been recognized as a most important resource of PUFA (**Narayan *et al.*, 2006, Simopoulos 1991**). EPA, which is the chief ingredient of omega-3 found in marine resource exhibits antibacterial activity against a spanned spectrum of bacteria (**Desbois *et al.*, 2008, Shin *et al.*, 2006)**. **Thompson *et al.*, (1994**) in their research on *Helicobacter pylori* concluded that inhibitory effects enhanced with the intensity of unsaturation, on the other hand, they did not go away from EPA in their unsaturation stage of fatty acids. Afterward, it was found that DHA has an advanced unsaturation level than EPA, was confirmed to have an

inhibitory outcome on gram-negative bacteria that surpasses that of EPA (**Shin *et al.* 2007)**.

2.3 Omega-3 in linseed

Flax or linseed (*Linum usitatissimum),* is one of the oldest saleable oils which has unsaturated (-CH=CH-) fatty acids like oleic acid (12–30%), linoleic acid (8–29%) and linolenic acid (35–67%) **(The Wealth of India 2006)**. Linseed oil has potentially realistic antibacterial properties as a current research. The hydrolyzed lipid has been situated successfully against *S. aureus* resistant to antibiotics. Bovine mastitis is a disease at dairy farms and is usually caused by bacterial infections, finally producing inflammation in the udder tissues **(Yagi, 2002)**. The anti-inflammatory action of linseed oil and antibacterial activity of hydrolyzed oil, it was considered meaningful to assess *L. usitatissimum* fixed oil for antimicrobial activity and therapeutic efficiency in bovine mastitis, an inflammatory disorder caused by microbial infection.

2.4 Role of *Staphylococcus aureus*

Staphylococcus aureus a Gram-positive coccus appears as grape-like clusters under microscopic observation and has bulky, round, golden-yellow colonies. *S. aureus* may occur in healthy persons as a commensal on the skin or in the nose and throat and, less commonly, may be found in the colon and in urine. Most *Staphylococcus* species are coagulase-negative, but *S. aureus* is coagulase-positive. This is medically important because *S. aureus* is much more aggressive and likely to be antibiotic-resistant **(Ryan, 2004)**. This is the reason that *S. aureus* constitutes the nearly everyone gets infected from common causes of *staphylococcus* infections. Infections due to *S. aureus* are a main source of morbidity and mortality worldwide and cover a range of illnesses, such as skin

infections and other fatal diseases, e.g., pneumonia, endocarditis, toxic shock syndrome (TSS) **(Kayser *et al.,* 2005)**, meningitis **(Pedersen *et al.,* 2006)** and septicemia **(Prasad, Mishra, & Pant, 2007)**. Scanty reports are available on silkworm pupal oil with reference to antimicrobial property. Hence, based on the constituents present in the pupal oil.

Silkworm pupal oil could be a tremendous lipid source for humans because of the presence of a 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 Silkworm pupal oil has numerous health benefits.

In the present study, mulberry tasar, eri and muga silkworm pupal oils were extracted and analyzed for its antibacterial and antifungal property against *Staphylococcus sciuri strain CD97* and *Phyllactinia corylea* strain.

2.5 Materials and Methods

2.5.1 Mulberry silkworm pupae

In order to get mulberry silkworm pupae oils, silkworm *Bombyx mori* the rearing was conducted according to **Krishnawami *et al.,* (1973**) method. For rearing 25 DFLs of silkworm egg were procured from NSSP (National Silkworm Seed Project), Central Silk Board, Bangalore for rearing purpose. The eggs were immediately incubated in the earthen pot for two days at 25°C. Further, the eggs were taken and put in the black boxing for uniform hatching of silkworms. After hatching the eggs, brushing was done with the help of feather. After brushing the larvae were fed with mulberry leaves till the

end of fifth instars. After spinning, cocoon was harvested and dried under sunlight. The pupae were taken out from the cocoon after the reeling.

2.5.2 Collection of non-mulberry silkworm pupae:

Whereas cocoons were directly procured from following places to extract oil from non- mulberry silkworms namely tasar, eri and muga.

1. Research extension centre (Tasar Central Silk Board, Ministry of Textiles, Govt. of India Bundelkhand University Jhansi Uttar Pradesh, India.
2. Pradesic Co-operative Sericulture Federation Limited Uttar Pradesh (Office of Assistant Director Sericulture Sonbhadra) Tasar silk Koya Market Dudhi Sonbhadra. Uttar Pradesh, India
3. Banvasi Seva Asram, Govindpur vaya Tura Sonbhadra, Uttar Pradesh, India
4. Director Central Muga Eri Research and Training Institute, Central Silk Board, Ministry of Textiles, Govt. Of India, Post Office - Lahdoigarh Jorhat, Assam (India) PIN -785700.

2.5.3 Preparation of silkworm dry pupae powder for the extraction of pupae oils

The present study, we cleaned dry pupae and grind into powder form with help of mortar and pestle. Further pupae powder of silkworms was extracted by using different solvents namely N-hexane, Petroleum ether or Di-methyl ether.

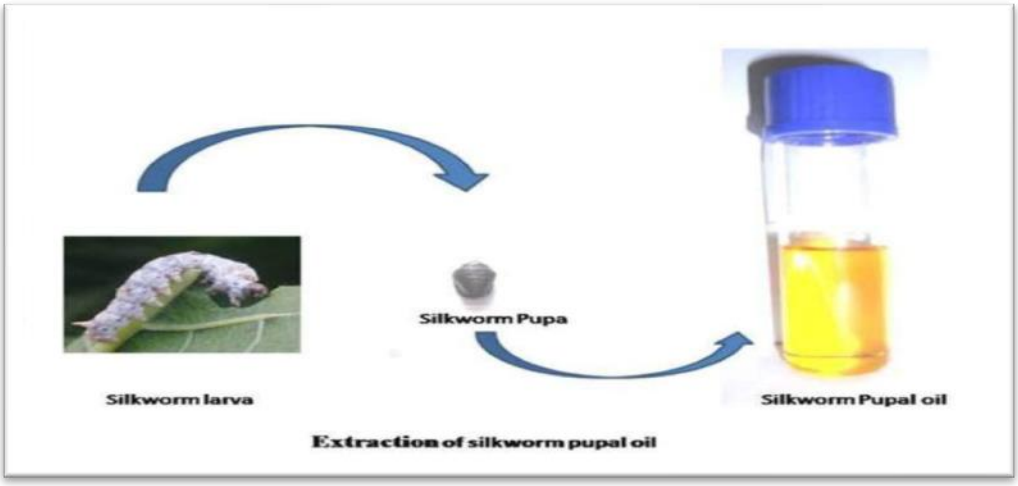
2.5.4 Extraction of pupae oils by maceration methods

The 10 g dry pupae powder of the Mulberry, Tasar, Eri and Muga silkworm were taken in the different reagent flask and in that flask about 30 ml of the solvent like N-hexane added. The solvent n-hexane was added in the reagent flask to that level so that

the level of the solvent was 1.5cm above the level of the pupa powder. The reagent flask was closed tightly with the cap and sealed with glycerin so that the solvent cannot be evaporated; since the solvent were used in the extraction of the oil was highly volatile and inflammable. The pupae powder and the solvent were kept for seven days, after that the solvent color changes from transparent to a yellowish, this shows that the solvent is ready for the extraction of oils.

Pic. 1. Silkworm Pupae

Pic. 2. Extraction of silkworm pupae oils



Pic. 3. Oils extraction from silkworm pupae

2.5.5 Extraction of mulberry silkworm pupal oils by using different solvents

The mulberry pupae oils were extracted by maceration method. For this 100 g of mulberry silkworm pupae powder added into 150 ml of different solvent separate, namely dimethyl ether, n-hexane and petroleum ether in order to find out the yield of mulberry oil with the different solvent. Further, both the mixtures of solvent and pupae powder maintain separately. When the solvent color changed to yellowish color then it was filtrated by the filter paper and the filtrate was filtered into the petri dish. Petri dish was kept in the open and shed room so that the volatile solvent in which the oil was extracted is evaporated and oils part was collected. In this extraction process of pupae oils the petroleum ether solvent gave higher amount of oils so, for further extraction of oils petroleum ether used as the solvent.

2.5.6 Extraction of non-mulberry silkworm pupae oils by using petroleum ether as solvent

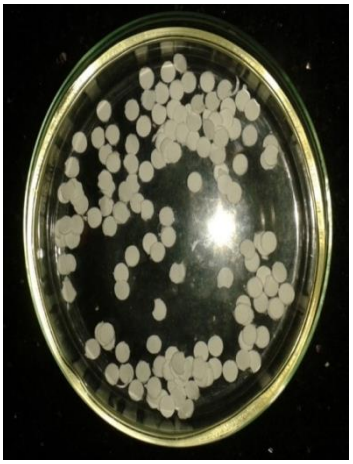
The oils of Tasar, Eri and Muga were extracted from their pupae by maceration method. 100 g of pupae powder of each variety were added separately into 150 ml of petroleum ether solvent. Further, both the mixtures of each variety were kept for seven days. Then filtered with filter paper and pupae oils were collected to find out the yield.

2.5.7 Antimicrobial activity of mulberry, tasar, eri and muga silkworm pupae oils

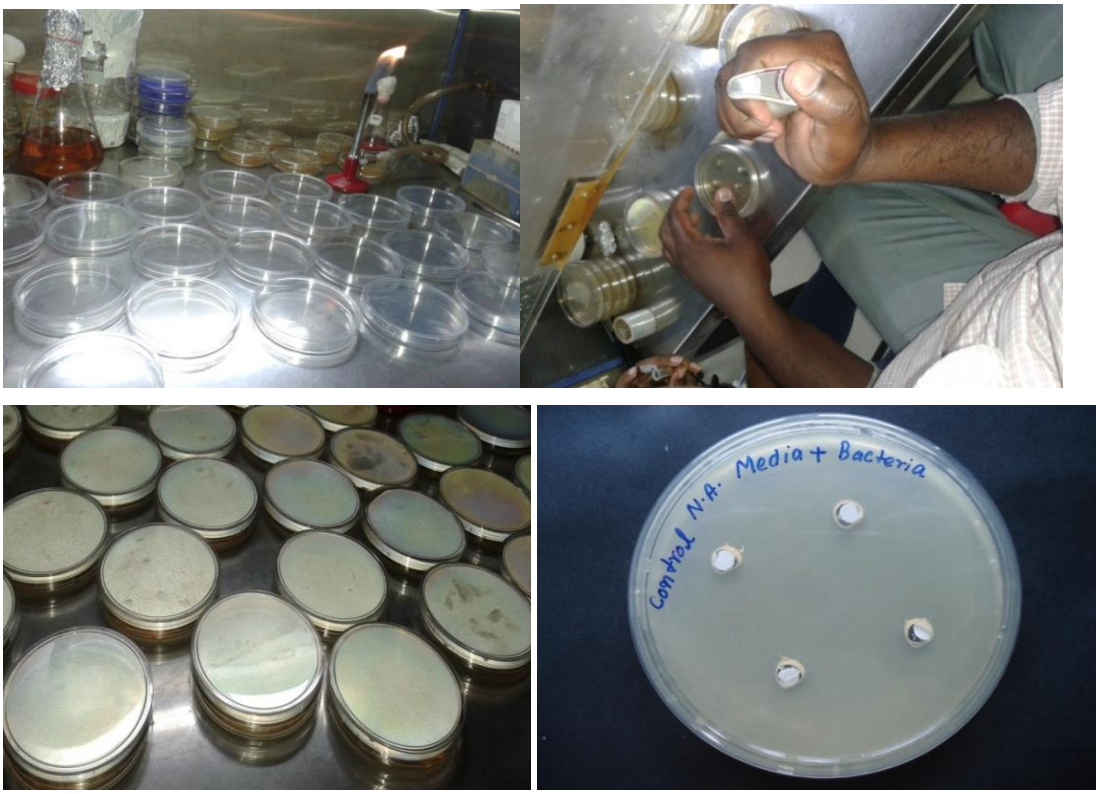
2.5.8 Materials

Infected mulberry leaves, distilled water, sterile water, 0.1% mercuric chloride solution, forceps, needles, Nutrient Agar plates and slants, PDA (Potato Dextrose Agar) plates and slants, 70% ethanol, tissue paper, beaker, inoculation loop, autoclave, laminar air flow cabinet.

Fic 4. Nutrient Agar Broth



Fic 5. Sterile disc of filter paper



Fic. 6. Bacterial culture plate preparation

In order to know the antibacterial and antifungal activity from different silkworm pupae oils of Mulberry, Tasar, Eri and Muga. The pathogenic bacteria S*taphylococcus sciuri* strain CD97 and fungus *Phyllactinia corylea* were isolated from the infected mulberry leaves.

Infected leaves were collected from mulberry garden Department of Applied Animal Sciences Babasaheb Bhimrao Ambedkar University (A Central University) Lucknow. The diseased part of samples were identified and a small part (2mm × 2mm) of it was cut off and washed with distilled water. It was then dipped in 0.1% mercuric chloride solution for two seconds for removing extracellular microorganisms. Washed thrice with sterile water to remove any traces of mercuric chloride. It was dried between tissue paper and placed in the center of the PDA (potato dextrose agar) for fungus and

NA (Nutrient Agar) for the bacterial plate. These plates were incubated at 26^0C (fungus) and 37^0C (bacteria) for 48 hours. Colonies were identified and repeatedly subculture to obtain their pure monoculture isolates. Slants were also made from pure culture plates. These pure culture plates and slants were stored in the refrigerator for future use. Laminar flow cabinet was sterilized by switching on U.V light for 15 minutes.

2.5.9 Further steps followed for molecular identification of bacteria:

To identify the Bacterial culture to its nearest species based on 16s r NA sequence.

2.5.9.1 Steps followed:

1. Genomic DNA was isolated from the cultural plate provided by the scientist (Dr. Venkatesh Kumar R).

2. The ~1.5kb rDNA fragment was amplified using high-fidelity PCR polymerase.

3. The PCR product was sequenced using the terminal primers, as well as an Intermediate primer, to cover the entire stretch of ~1.5Kb.

4. The sequence data was aligned and analyzed to identify the bacterium and its Closest neighbors.

2.5.9.2 Results

1. The microbe was found to be most similar to Uncultured *Staphylococcus sp. clone CD33 16S ribosomal RNA gene, partial sequence*

(NCBI Accession No: gb|KF760554.1|

2. The next closest homologue was found to *be Staphylococcus sciuri strain CD97* 16S ribosomal RNA gene, partial sequence (NCBI Accession No: gb|JX871318.1|

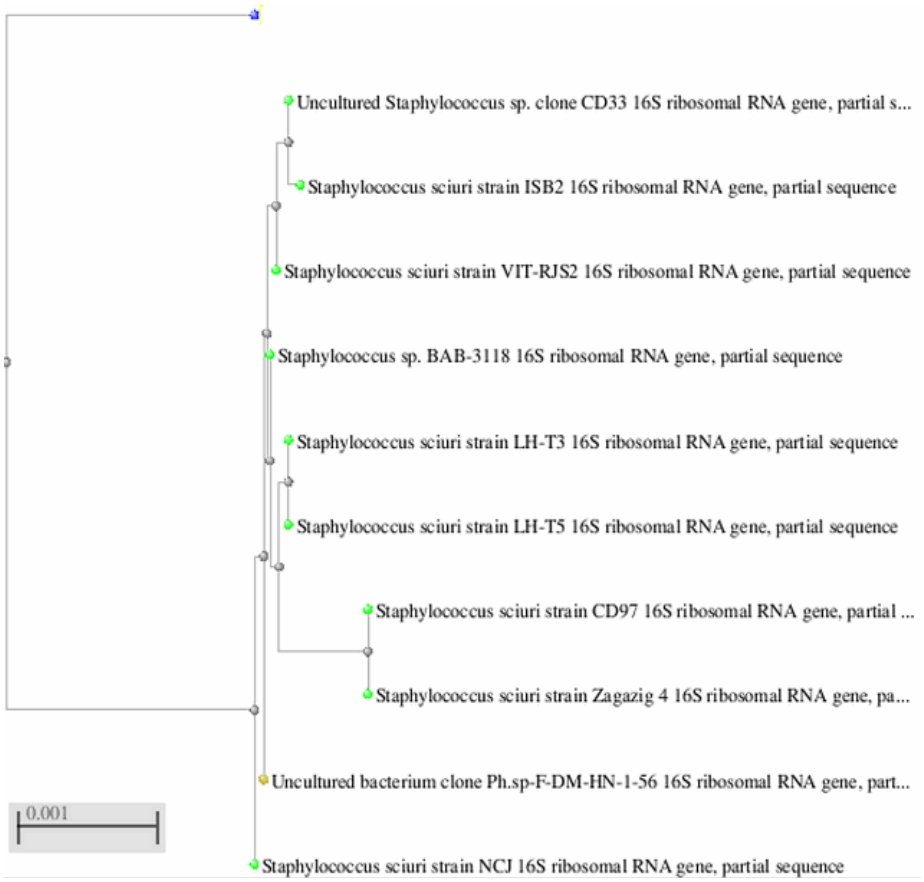
2.5.9.3 Aligned Data:

ACCCAGATCGGGTCTGGACTACGCGCTGCCTATAATGCAGTCGAGCGAACAGATGAGAAGCT
T

GCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGG
A
TAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACCGCATGGTTCAATAGTGAAA
G
ACGGTTTCGGCTGTCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGC
T
TACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACAC
G
GTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGA
G
CAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAA
TT
TGTTAGTAACTGAACAAGTCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
C
AGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAG
G
CGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGA
A
ACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGG
A
GGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGG
G
GATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGG
G
GGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCA
A
GGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
AAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACCGCTCTAGAGATAGAGTCTTC
CC
CTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
T
TAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAGGT
T
GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGAT
TT
GGGCTACCACACGTTGTTTCCAAGGATTAAACACAAAGGGCAGCGAATTCCGCGAGGCCAAG
C

AAATCCCATAAAATTATTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAA
T
CGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCC
G
TCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAAGTAACCTTTAGGAGCTAAGCCGT
C
GAAGAGGACAGTTGATCCCCTTTATTTGCTGT

2.5.9.4 PHYLOGENETIC TREE



2.5.9.5 Other relavent information

1. Genomic DNA was isolated from the culture provided using Chromous Genomic DNA solation kit

2.5.9.6 Gel Photo

Fig 1: Extraction of Genomic DNA from Bacterial sample using the Bacterial Genomic DNA Isolation Kit (RKN15).

2. PCR cycling Parameters:

• Name of the Thermal Cylcer: ABI2720

2.5.9.7 PCR Amplification conditions:

DNA: 1 µl

16s Forward Primer 400ng

16s Reverse Primer 400ng

dNTPs (2.5mM each) 4 µl

10X Taq DNA Polymerase Assay Buffer 10 µl

Taq DNA Polymerase Enzyme (3U/ µl) 1 µl

Water X µl

Total reaction volume: 100 µl

All PCR reagents were of Chromous Make.

2.5.9.8 Other relavent information:

1. Genomic DNA was isolated from the culture provided using Chromous Genomic DNA isolation kit

2.5.9.9 Gel Photo:

Fig 1: Extraction of Genomic DNA from Bacterial sample using the Bacterial Genomic DNA Isolation Kit (RKN15).

2. PCR cycling Parameters:

• Name of the Thermal Cylcer: ABI2720

2.5.9.10 PCR Amplification conditions:

DNA: 1 µl

16s Forward Primer 400ng

16s Reverse Primer 400ng

dNTPs(2.5mM each) 4 µl

10X Taq DNA Polymerase Assay Buffer 10 µl

Taq DNA Polymerase Enzyme (3U/ µl) 1 µl

Water X µl

Total reaction volume: 100 µl

All PCR reagents were of Chromous Make.

94°C	94°C	55°C	72°C	72°C
5min	30sec	30sec	130min	7min
	35 cycles			

3. Forward and reverse primer sequence which was used for amplification of 16s rDNA sequence:

2.5.9.11 Prokaryotes: 16s rRNA specific primer

16s Forward Primer:

5'-AGAGTRTGATCMTYGCTWAC-3'

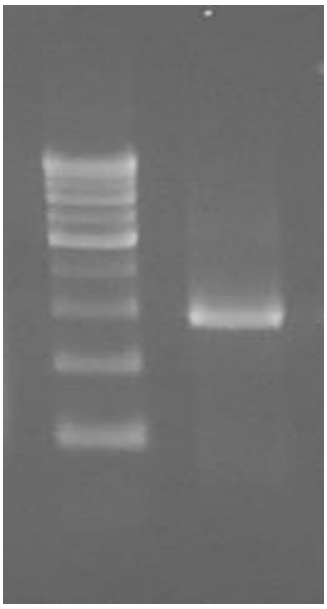
16s Reverse Primer:

5’-CGYTAMCTTWTTACGRCT-3’

The PCR product size 1.5kb:

4. PCR amplified 16s rDNA Gel doc picture with ladder:

Fig 2: PCR amplification of 16s rDNA fragment from Bacterial sample. The size of PCR amplified product is ~1.5kb

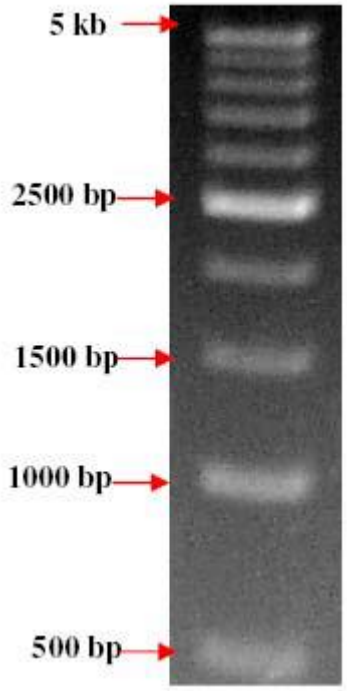


2.5.9.12 Lane description:

L: 500bp DNA Ladder (Chromous Cat. No. LAD02)

1: PCR product of Bacillus

2.5.9.13 500bp DNA Ladder (LAD02):



500 bp ladder contains 10 DNA fragments of size 500 bp, 1000 bp, 1500 bp, 2000 bp, 2500 bp, 3000 bp, 3500 bp, 4000 bp, 4500 bp and 5000 bp

2.5.9.14 Sequencing Reaction:

2.5.9.14.1 The Sequencing mix Composition and PCR Conditions are as follows:

2.5.9.14.2 (i) 10µl Sequencing Reaction

• Big Dye Terminator Ready Reaction Mix: 4µl

• Template (100ng/ul):1µl

• Primer (10pmol/λ):2µl

• Milli Q Water: 3µl

2.5.9.14.3 (ii) PCR Conditions: (25 cycles)

Initial Denaturation: 96°C for 5 min Denaturation: 96°C for 30 sec

Hybridization: 50 °C for 30 sec

Elongation: 60 °C for 1.30 min

2.5.9.14.4 (iii) Instrument and Chemistry Details

Sequencing Machine: **ABI 3130 Genetic Analyzer**

Chemistry: **Big Dye Terminator version 3.1**"

Cycle sequencing kit.

Polymer &

Capillary Array**: POP_7 polymer**

50 cm Capillary Array.

Analysis protocol: **BDTv3-KB-Denovo_v 5.2**

Data Analysis: **Seq Scape_ v 5.2**

Software

Reaction Plate: Applied Biosystem Micro Amp

2.5.9.14.5 (iv) Optical 96-Well Reaction plate

7. Identification software details:

Phylogentic Tree Builder uses sequences aligned with System Software aligner. A distance matrix is generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weighbor with alphabet size 4 and length size 1000.

2.5.9.15 Weighbor Tree: Weighbor is a weighted version of Neighbor-Joining that gives significantly less weight to the longer distances in the distance matrix. The weights are based on variances and covariances expected in a simple Jukes-Cantor model.

2.5.9.16 Jukes-Cantor Correction: The Jukes-Cantor distance correction is a model which considers that as two sequences diverge, the probability of a second substitution at

any nucleotide site increases. For distance-based trees such as Weighbor, the difference in nucleotides is considered for the distance, therefore, second substitutions will not be counted and the distance will be underestimated. Jukes and Cantor created a formula that calculates the distance taking into account more than just the individual differences (1969; *Evol. of Protein Molecules*, Academic Press)

2.5.9.17 Bootstrap: Bootstrapping is a statistical method for estimating the sampling distribution by resampling with replacement from the original sample. In making phylogenetic trees, the approach is to create a pseudo alignment by taking random positions of the original alignment. Some columns of the alignment could be selected more than once or not selected at all. The pseudo alignment will be as long as the original alignment and will be used to create a distance matrix and a tree. The process is repeated 100 times and a majority consensus tree is displayed showing the number (or percentage) of times a particular group was on each side of a branch without concerning the subgrouping.

2.5.10 Identification of fungal strain isolated from mulberry leaves

Dr. Rajesh Kumar Professor and head of Department of Microbiology, Babasaheb Bhimrao Ambedkar University (A Central University) Lucknow, identified the fungal strain collected from the mulberry garden. The strain was *Phyllactinia corylea.* Powdery mildew caused by *Phyllactinia corylea* (Pers.) Karst is a serious disease of mulberry inflicts significantly qualitative as well as quantitative losses. Owing to non eco-friendly nature, toxicity to silkworm, high cost and other side effects of chemical control measures, search for other managing strategy devoid of such drawbacks become expected. Powdery mildew of mulberry caused by *Phyllactinia corylea* (Pers.) Karst.

reduces leaf yield and adversely affects the feeding quality of the leaf due to luxuriant mycelial growth on lower surface (**Noamani *et al.*, 1970**).

Fic.7. Mulberry leaves infected with *Phyllactinia corylea*

Fic.8 http://agritech.tnau.ac.in/sericulture/disese%20mgt_mulberry.html

2.5.11After identification of bacterial and fungal strain screening of antifungal and antibacterial

2.5.12 Screening for antimicrobial activity

The oils extracted from both mulberry and non-mulberry (Tasar, Eri and Muga) of silkworm pupae were screened against pathogenic bacteria (*Staphylococcus sciuri* strain CD97) and fungi (*Phyllactinia* corylea) by Disc Diffusion and MIC (Minimum Inhibitory Concentration) method.

2.5.13 Antifungal activity by Disc Diffusion Methods:

Antifungal activity was tested by the disc diffusion method (**Meena *et al.*, 1994**). In disc diffusion method the fungal suspensions were prepared to identify the growth of fungal strains on potato dextrose agar media, and Whatman sterile filter paper (6 mm diameter) was placed into the wells of potato dextrose agar plate. Mulberry and non-mulberry

silkworm pupae oils were kept in the filter disc in different concentration, i.e. 25%, 50%, 75%, and 100% in the same manner one plate kept as control. Different concentrations were made by using sterile glycerin which is a non-polar solvent. All the plates were incubated at 28^0C for 48 hours. After incubation, the antifungal activity was evaluated by measuring the growth of bacterial strains. The zones of inhibition were measured by digital vernier caliper.

2.5.14 Antibacterial activities by Disc Diffusion Methods:

Antibacterial activity was tested by the disc diffusion method (**Meena *et al.*, 1994**). In disc diffusion method the bacterial suspensions were prepared to identify the growth of bacterial strains on nutrient agar media, and Whatman sterile filter paper (6 mm diameter) was placed into the wells of the nutrient agar plate. Mulberry and non-mulberry silkworm pupae oils were kept in the filter disc in different concentration, i.e. 20%, 40%, 60%, 80% and 100% in the same manner, one plate kept as control. Different concentration was made by using sterile glycerin which is a non-polar solvent. All the plates were incubated at 37^0C for 48 hours. After incubation, the antibacterial activity was evaluated by measuring the growth of bacterial strains. The zones of inhibition were measured by digital vernier caliper.

2.5.15 Antibacterial and antifungal activities of the mulberry and non- mulberry silkworm pupae oils expressed as minimum inhibitory concentrations (MIC)

The MIC test was done by using **(Fuente *et al.*, 2006)** method. The media to MIC test were used as Muller Hinton Broth (MHB). The bacterial suspensions were prepared to identify the growth of bacterial strains *Staphylococcus sciuri* strain CD97 in MHB media. The tests were conducted in **forty test tubes**: Following method used to the screening of antibacterial activity by MIC method:

In one group five test tubes were arranged in which one test tube for control, four test tubes were with bacterial suspension in which oils of **mulberry** silkworm pupal oils added at a different concentration of 12.5, 25, 50, and 100 µl/ml respectively. Another five test tube was arranged in which one test tube was for control, four test tube were for bacterial suspension with DMSO (Dimethyl Sulfoxide) in which antibiotic streptomycin added at the different concentration of 12.5, 25, 50, 100 µl/ml respectively.

In second group five test tubes were arranged in which one test tube for control, four test tubes were with bacterial suspension in which oils of **tasar** silkworm pupae oils added at the different concentration of 12.5, 25, 50, and 100 µl/ml respectively. Another five test tube was arranged in which one test tube was for control, four test tube was for bacterial suspension with DMSO in which antibiotic streptomycin added at a different concentration of 12.5, 25, 50, 100 µl/ml respectively.

In third group, five test tubes were arranged in which one test tube for control; four test tubes were with bacterial suspension in which oils of eri silkworm pupae oils added at different concentration of 12.5, 25, 50, and 100 µl/ml respectively. Another five test tube was arranged in which one test tube was for control, four test tube was for bacterial suspension with DMSO in which antibiotic streptomycin added at different concentration of 12.5, 25, 50, 100 µl/ml respectively.

In fourth group five test tubes was arranged in which one test tube for control, four test tubes was with bacterial suspension in which oils of muga silkworm pupal oils added at different concentration of 12.5, 25, 50, and 100 µl/ml respectively. Another five test tube was arranged in which one test tube was for control, four test tube were for

bacterial suspension with DMSO in which antibiotic streptomycin added at different concentration of 12.5, 25, 50, 100 µl/ml respectively.

All bacterial and fungal test tubes were incubated at 37^0C and 30^0C for 72 and 48 hours. After incubation, OD (optical density) was taken at 610 nm by spectrophotometer.

2.6 Results:

Extraction of mulberry and non mulberry silkworm pupae oils were done by using a different solvent like Di-methyl ether, N-hexane and petroleum ether showed in table 1and figure 2. After extraction of pupae oils by using different solvents it was found that the petroleum ether solvent extracted more oil than Di-methyl ether and N-hexane. So, further extraction processes were done by Petroleum ether.

Extraction of non-mulberry (Tasar, Eri and Muga) silkworm pupae oils by using petroleum ether the eri silkworm pupae oils given higher amount than muga and tasar silkworm pupae oils showed in table 2 and figure 3.

2.6.1 Extraction of mulberry silkworm pupae oils by using three different solvents as: Di-methyl Ether, N-Hexane, Petroleum Ether

Table 1. Extraction of mulberry silkworm pupae oils by using different solvents

SL No.	Name of Solvent	Mulberry Pupae powder (in gm)	Solvent used (in ml)	Yield of oil obtained (in ml)
1	Di-methyl Ether	100g	150ml	1.0ml
2	N-Hexane	100g	150ml	1.5ml
3	Petroleum Ether	100g	150ml	3.5ml

Table 2. Extraction of non mulberry pupae oils by using Petroleum ether solvent

SL No.	Silkworm Race	Solvent Used	Pupae powder of silkworm (in gm)	Solvent used (ml)	Yield of oil obtained (ml)
1.	Eri	Petroleum Ether	100g	150ml	3.6ml
2.	Tasar	Petroleum Ether	100g	150ml	1.8ml
3.	Muga	Petroleum Ether	100g	150ml	2.5ml

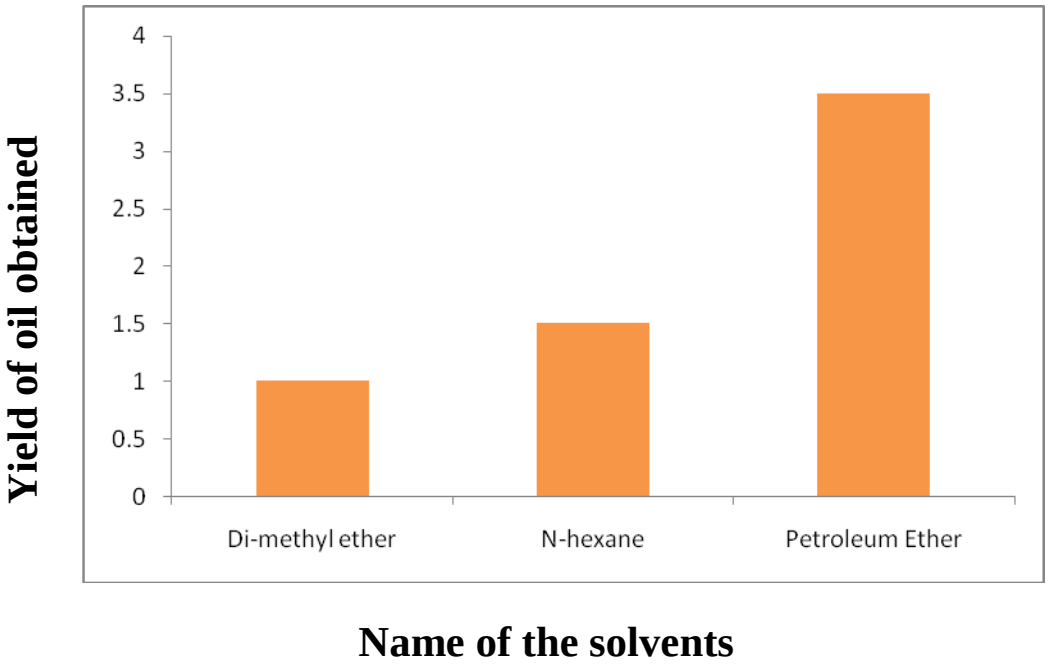


Fig 2. Yield of Mulberry Silkworm pupal oil by using different solvent

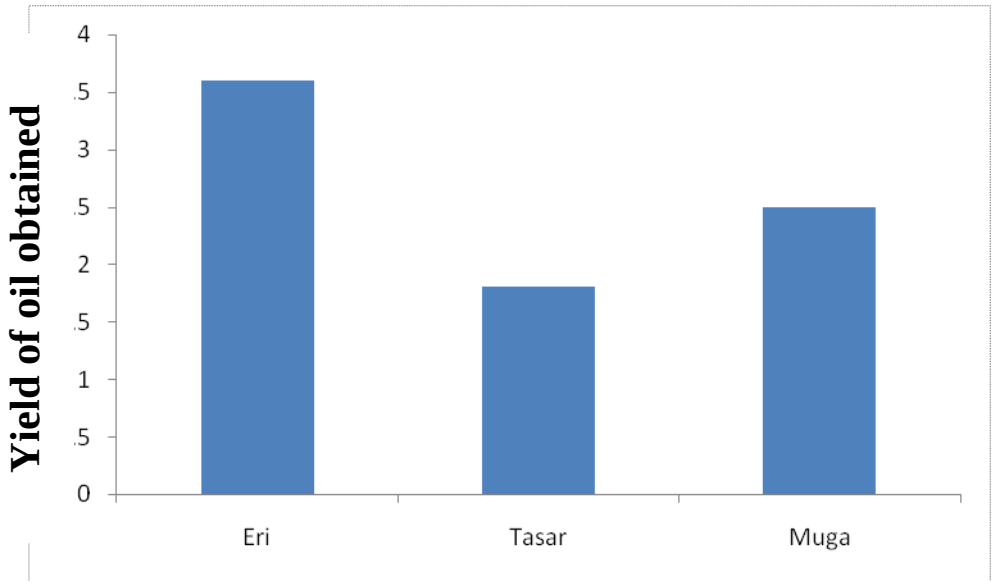
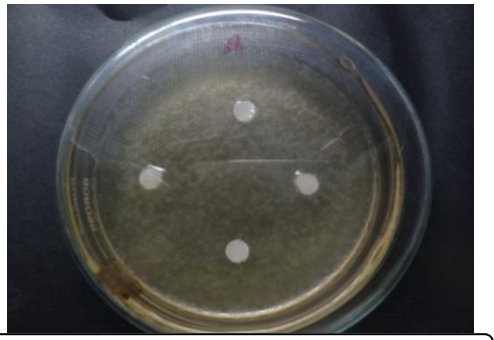


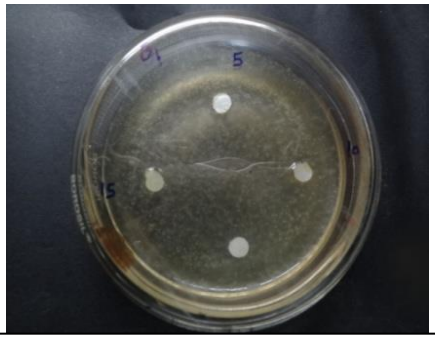
Fig 3. Oil yield of non-mulberry silkworm pupae by petroleum ether solvents

2.6.2 Antifungal activities of mulberry and non-mulberry silkworm pupae oils by disk diffusion method:

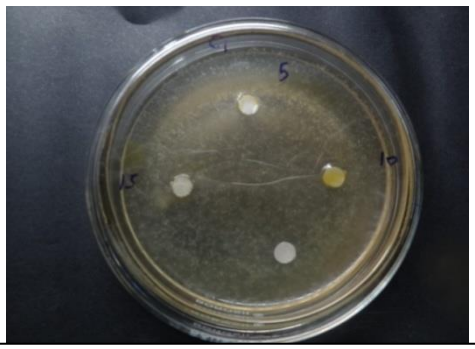
The fungus powdery mildew (*Phyllactinia* corylea) isolated from mulberry leaves of diseased plants were highly resistant against mulberry and non-mulberry silkworm pupae oils and hence no antifungal activity was shown in picture 9.



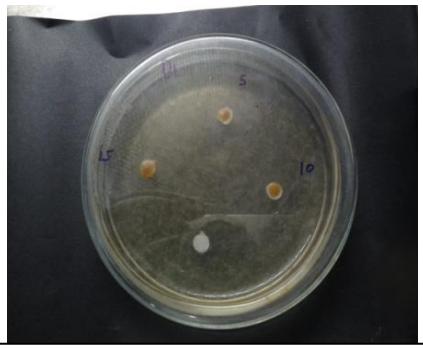
Mulberry pupae oils 25%, 50%, 75%, 100%



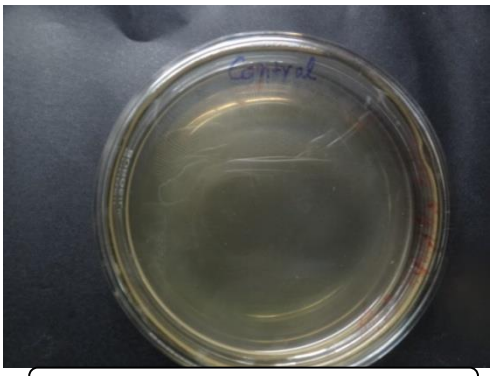
Tasar pupae oils 25%, 50%, 75%, 100%



Eri pupae oils 25%, 50%, 75%, 100%



Muga pupae oils 25%, 50%, 75%, 100%

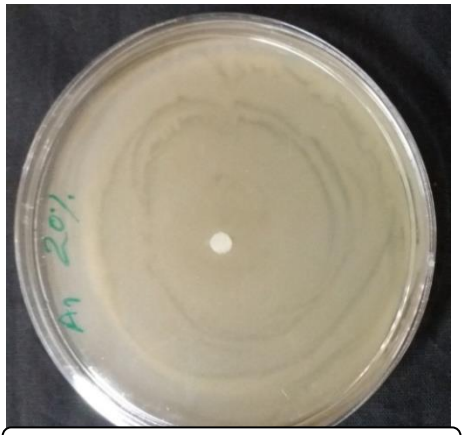


Control

Fic.9. Silkworm pupae oils not affecting on isolated mulberry leaves fungus

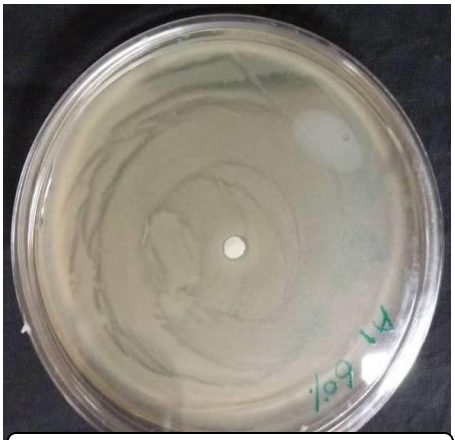
2.6.3 Antibacterial activities of mulberry and non-mulberry silkworm pupae oils by disk diffusion method:

3.1a Antibacterial activity of Mulberry silkworm pupae oils:

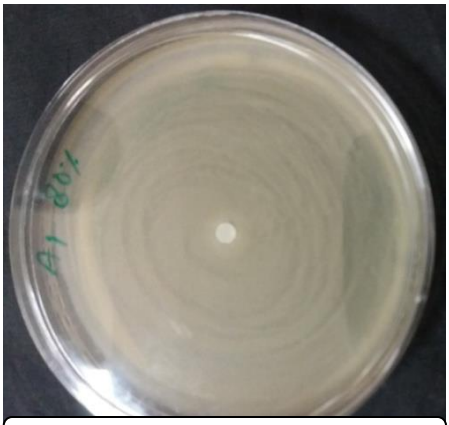


Mulberry pupae oils 20%

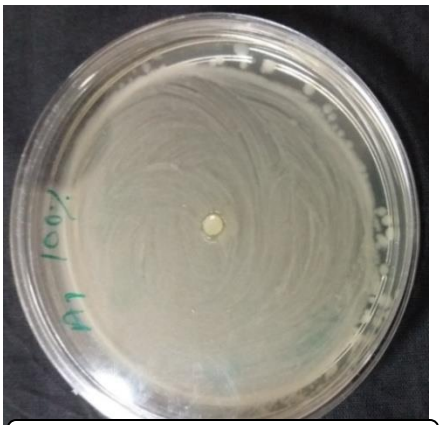
Mulberry pupae oils 40%



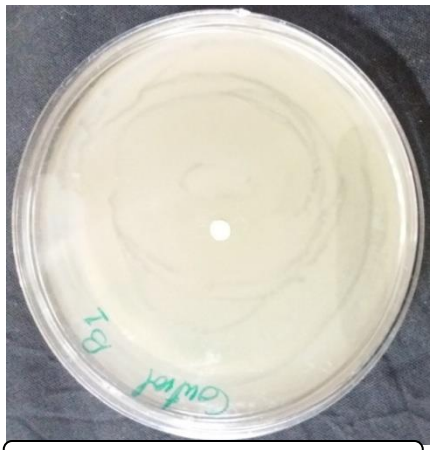
Mulberry pupae oils 60%



Mulberry pupae oils 80%



Mulberry pupae oils

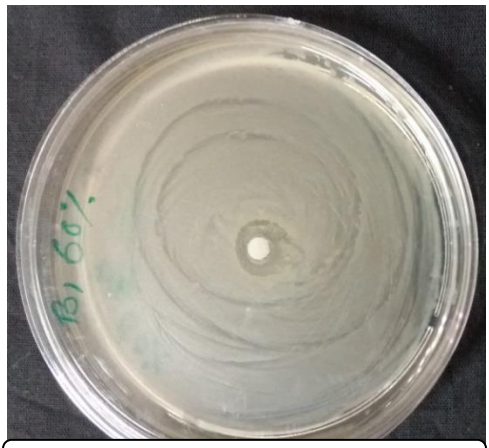


Control

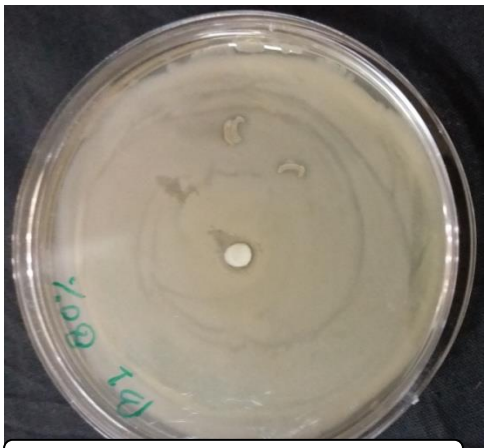
3.1b Antibacterial activity of Tasar silkworm pupae oils:

Tasar pupae oils

Tasar pupae oils

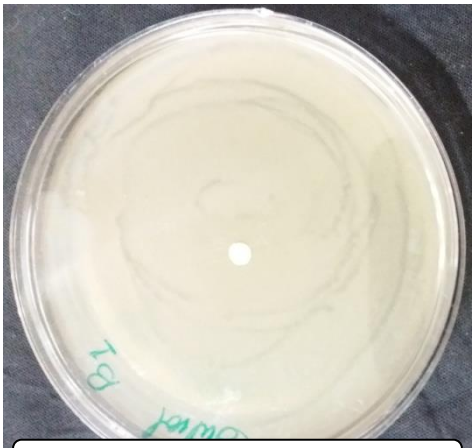


Tasar pupae oils



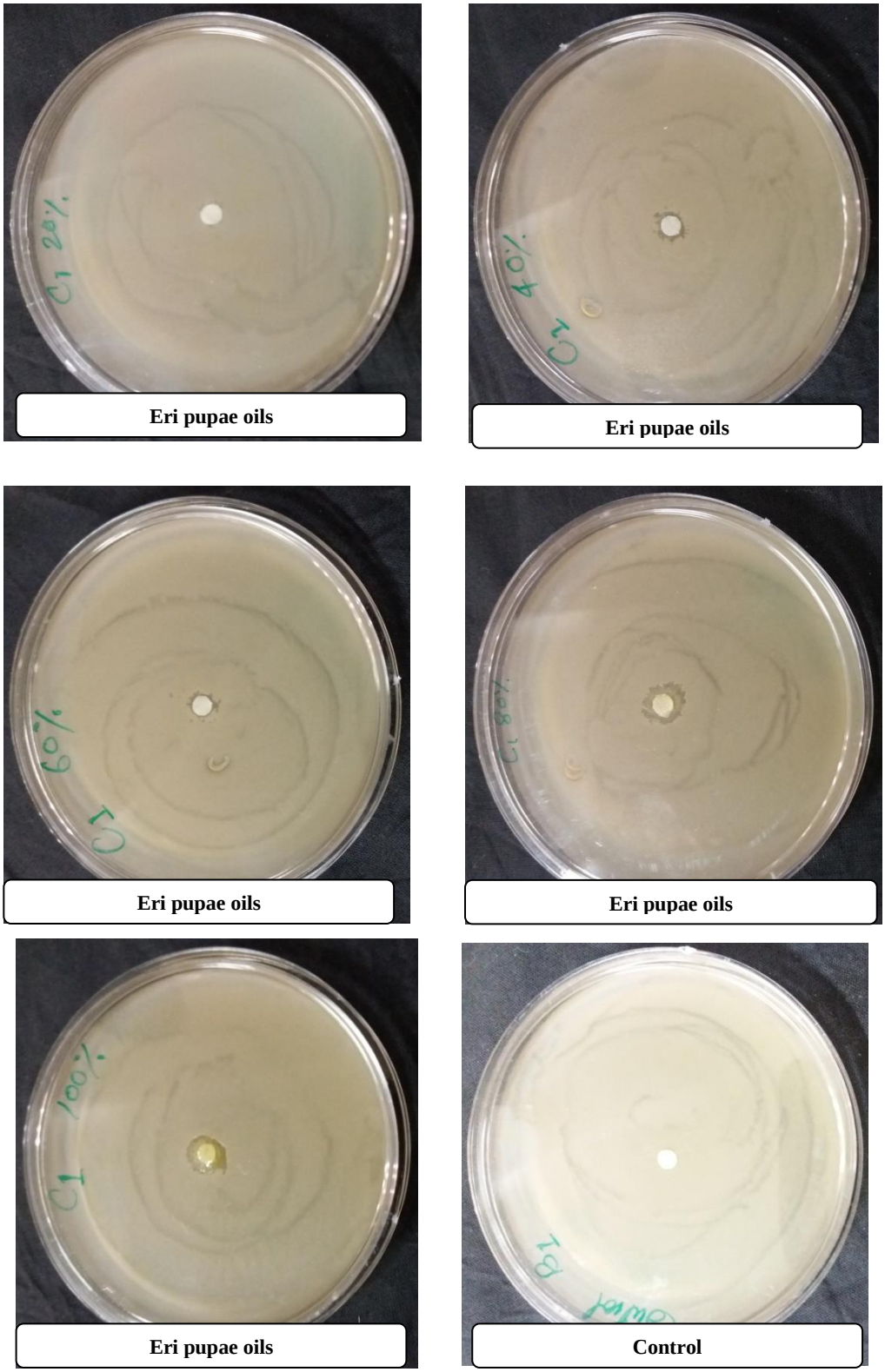
Tasar pupae oils

Tasar pupae oils

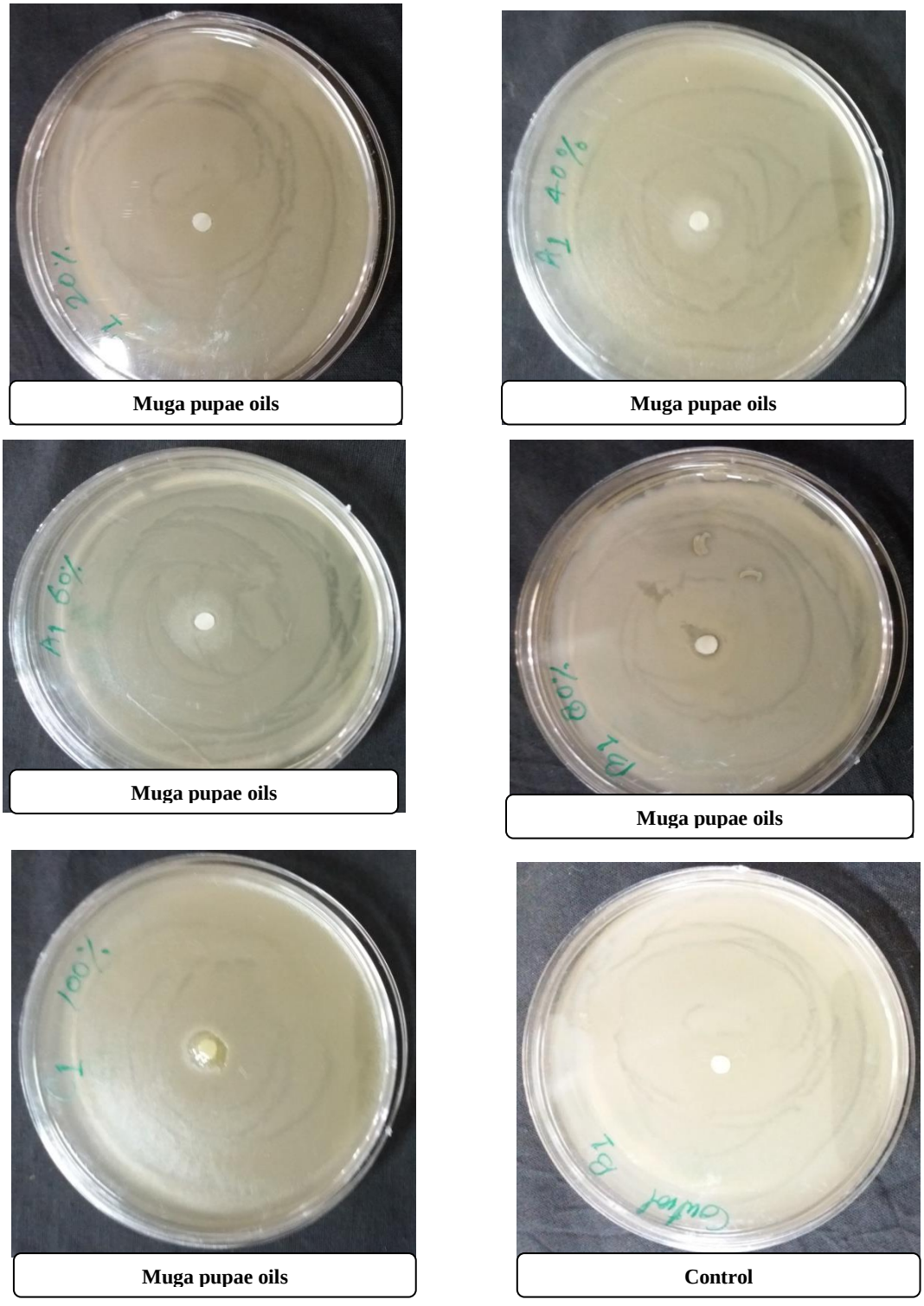


Control

3.1c Antibacterial activity of Eri silkworm pupae oils:



3.1d Antibacterial activity of Muga silkworm pupae oils



Fic 10. Antibacterial activity of mulberry and non mulberry silkworm pupal oil on *Staphylococcus sp.*

Table: 3. Zone of inhibition of mulberry and non mulberry silkworm pupae oils at 24 hours.

Treatment	24 Hours				
	20%	**40%**	**60%**	**80%**	**100%**
Mulberry	7.72 ± 0.447	8.78 ± 0.468	9.98 ± 0.247	11.15 ± 0.495	12.13 ± 0.318
Tasar	5.75 ± 0.144	6.25 ± 0.144	8.63 ± 0.125	9.25 ± 0.323	10.87 ± 0.698
Eri	6.00 ± 0.204	6.38 ± 0.315	7.38 ± 0.125	7.94 ± 0.616	10.75 ± 0.520
Muga	6.19 ± 0.449	7.05 ± 0.289	8.38 ± 0.375	9.85 ± 0.645	10.96 ± 0.559
CV%	10.623	9.041	5.620	11.205	9.685
LSD $_{0.05}$	1.272	1.236	1.070	1.594	1.603
LSD $_{0.01}$	1.783	1.733	1.501	2.234	2.247

Table: 4. Zone of inhibition of mulberry and non mulberry silkworm pupae oils at 72 hours.

Treatment	72 Hours				
	20%	**40%**	**60%**	**80%**	**100%**
Mulberry	9.110 ±0.152	10.563 ±0.577	12.513 ±0.296	13.000 ±0.456	14.125 ±0.315
Tasar	8.125 ±0.125	9.375 ±0.239	10.000 ±0.204	11.313 ±0.277	12.875 ±0.591
Eri	8.250 ±0.250	9.000 ±0.204	9.813 ±0.277	10.125 ±0.582	12.625 ±0.473
Muga	8.750 ±0.250	9.838 ±0.356	11.313 ±0.344	12.625 ±0.239	13.750 ±0.250
CV%	4.729	7.712	5.223	7.014	6.422
LSD $_{0.05}$	0.980	1.332	1.163	1.400	1.426
LSD $_{0.01}$	1.374	1.868	1.631	1.962	2.000

The bacterial species had shown significant zone of inhibition in case of mulberry silkworm pupae oils at 100%, 12.13 ± 0.318 mm and at 100%, 14.125 ±0.315 mm at 24

and 72 hour respectively when compared to other pupae oils showed in table 3, 4 and picture 10.

2.6.4 Antibacterial activity of mulberry and non-mulberry silkworm pupal oils by Minimum Inhibitory Concentration (MIC) method:

Table. 5. MIC of mulberry and non mulberry silkworm pupae oils on *Staphylococcus sciuri strain CD97*

Treatment	Mulberry pupal oil	Tasar pupal oil	Eri pupal oil	Muga pupal oil
Control	0.907±0.005	0.311±0.009	0.556±0.0005	0.950±0.0010
12.5µl	0.887±0.002	0.295±0.005	0.508±0.0005	0.818±0.0005
25 µl	0.787±0.0002	0.183±0.005	0.254±0.0037	0.716±0.0054
50 µl	0.705±0.057	0.168±0.006	0.238±0.0010	0.624±0.0056
100 µl	0.714±0.060	0.164±0.011	0.395±0.0015	0.850±0.0361
CV%	0.066	0.014	0.003	0.029
LSD $_{0.05}$	0.092	0.019	0.004	0.041
LSD $_{0.01}$	4.641	3.550	0.490	2.088

2.6.5 MIC of Mulberry Silkworm Pupae oils:

The tests were conducted in five test tubes for test sample in which control as media with bacteria to whom mean±SD was 0.907±0.005, and test sample made as media, bacteria with mulberry silkworm pupae oils (MSPO) at different concentration of 12.5, 25, 50, 100 µl/ml and CV%, $LSD_{0.05}$, $LSD_{0.01}$which mean±SD values were 0.887±0.002, 0.787±0.0002, 0.705±0.057, 0.714±0.06 and 0.066, 0.092, 4.641 respectively. Another five test tubes with positive control as media with bacteria which

mean±SD was 0.311±0.005, and positive control made as media, bacteria and antibiotic streptomycin at different concentration of 12.5, 25, 50 and 100 µl/ml respectively which mean±SD values were 0.139±0.006, 0.084±0.005, and 0.018±0.003 0.016±0.005 respectively showed in table 6 and figure 4, 5, 6 and 7.

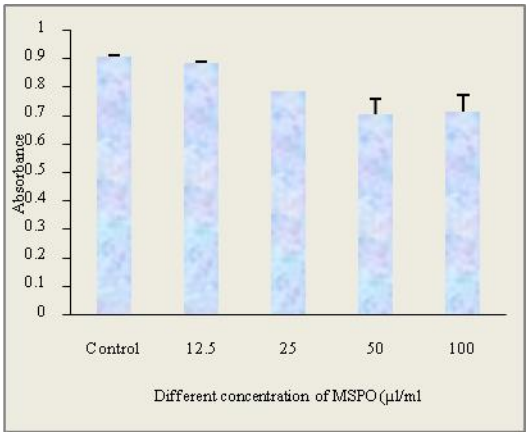


Fig..4. MIC of mulberry silkworm pupal oil (MSPO) on *Staphylococcus sciuri strain CD97*

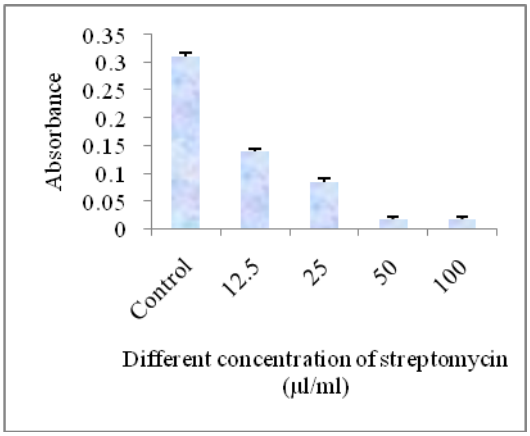


Fig.5. MIC of streptomycin on *Staphylococcus sciuri strain CD97*

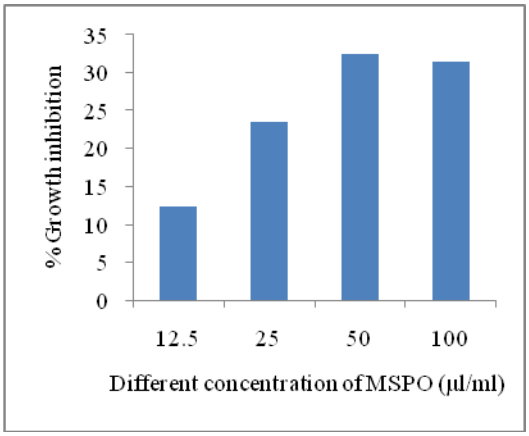


Fig.6. Percentage growth inhibition of mulberry silkworm pupal oil on *Staphylococcus sciuri strain*

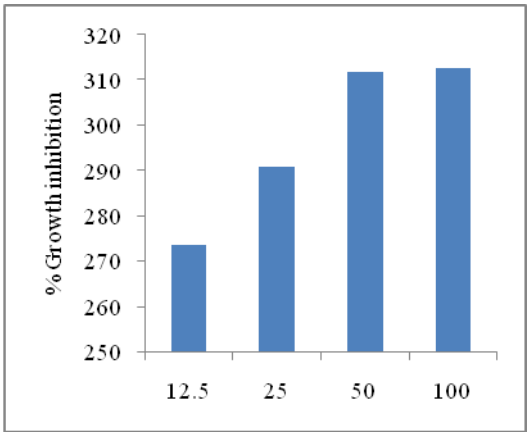


Fig.7. Percentage growth inhibition of streptomycin on *Staphylococcus sciuri strain CD97*

Table: 6. Minimum inhibitory concentration of mulberry silkworm pupae oils, streptomycin and percentage growth inhibition on *Staphylococcus sciuri strain CD97*

Concentration (µl/ml)	Mulberry pupal oil Mean±SD (OD)	% Growth inhibition by Mulberry Pupae oils	Streptomycin Mean±SD (OD)	% Growth inhibition by Streptomycin
Control	0.907±0.005	-	0.311±0.005	-
12.5	0.887±0.002	12.37	0.139±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	312.49

2.6.6 MIC of Tasar Silkworm Pupal Oils:

The tests were conducted in five test tubes with control as media with bacteria which mean±SD was 0.311±0.009, and test sample made as media, bacteria with tasar silkworm pupal oil (TSPO) at different concentration of 12.5, 25, 50 and 100 µl/ml and CV%, $LSD_{0.05}$, $LSD_{0.01}$ respectively, which mean±SD values were 0.295±0.005, 0.183±0.0005, 0.168±0.006, 0.164±0.011 and 0.014. 0.019, 3.550 respectively. Another five test tubes with positive control as media with bacteria which mean±SD was 0.325±0.0014, and positive control made as media, bacteria and the antibiotic streptomycin at different concentration of 12.5, 25, 50 and 100 µl/ml, respectively which mean±SD values were 0.139±0.004, 0.082±0.007, 0.0158±0.0005, and 0.013±0.001 respectively showed in table 7 and figure 8, 9, 10 and 11.

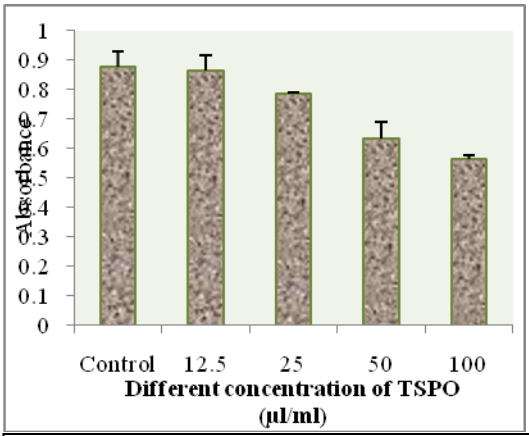


Fig.8.. MIC of tasar silkworm pupal oil on *Staphylococcus sciuri strain CD97*

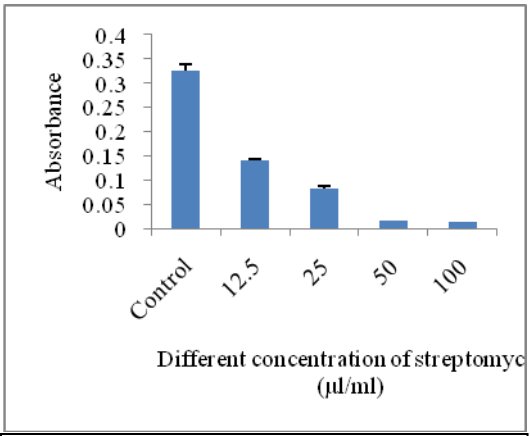


Fig 9. MIC of streptomycin on *Staphylococcus sciuri strain CD97*

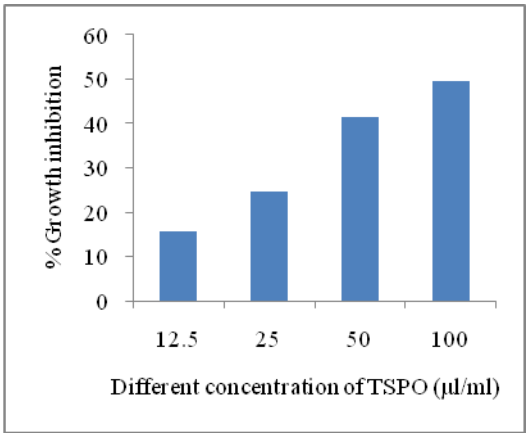


Fig10.. Percentage growth inhibition of tasar silkworm pupal oil on *Staphylococcus sciuri strain CD97*

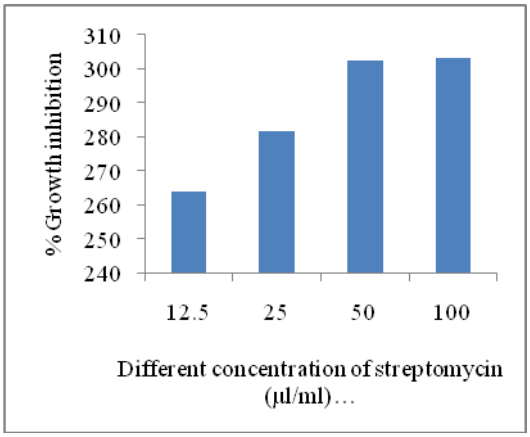


Fig.11. Percentage growth inhibition of streptomycin on *Staphylococcus sciuri strain*

Table: 7. Minimum inhibitory concentration of tasar silkworm pupae oils, streptomycin and percentage growth inhibition on *Staphylococcus sciuri strain CD97*

Concentration (µl/ml)	Tasar pupal oil Mean±SD (OD) nm	% Growth inhibition by Tasar Pupae oils	Streptomycin Mean±SD (OD) nm	% Growth inhibition by Streptomycin
Control	0.311±0.009	-	0.325±0.014	-
12.5	0.295±0.005	15.7086	0.139±0.004	264.256
25	0.183±0.005	24.619	0.0824±0.0071	281.904
50	0.168±0.006	41.557	0.0158±0.0005	302.342
100	0.164±0.011	49.62	0.0135±0.0011	303.066

2.6.7 MIC for eri silkworm pupae oils

The tests were conducted in five test tubes with control as media with bacteria which mean±SD was 0.556±0.0005, and test sample made as media, bacteria with eri silkworm pupal oil (ESPO) at different concentration of 12.5, 25, 50, 100µl/ml and CV%, $LSD_{0.05}$, $LSD_{0.01}$ which mean±SD values were 0.508±0.0005, 0.254±0.0037, 0.238±0.001, 0.395±0.0015 and 0.003, 0.004, 0.490 respectively. Another five test tubes with positive control as media with bacteria which mean±SD was 0.556±0.0005, and positive control made as media, bacteria and the antibiotic streptomycin at different concentration of 12.5, 25, 50 and 100 µl/ml, respectively which mean±SD values were 0.328±0.010, 0.232±0.159, 0.138±0.001, and 0.095±0.001 respectively showed in table 8 and figure 12, 13, 14 and 15.

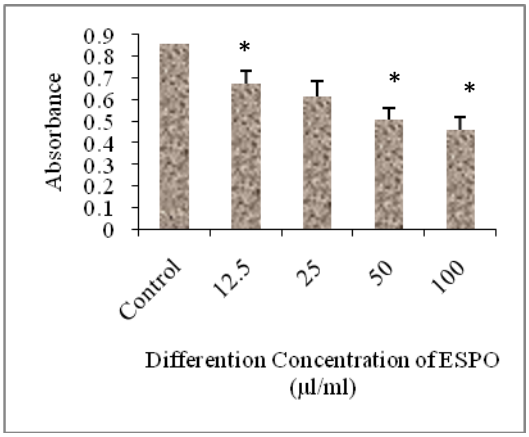


Fig.12. MIC of eri silkworm pupal oil on *Staphylococcus sciuri strain CD97*

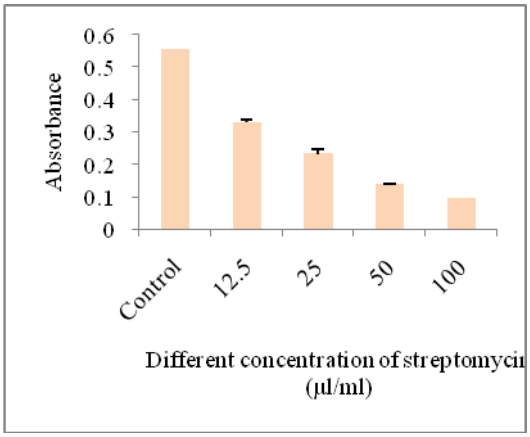


Fig.13. MIC of sterptomycin on *Staphylococcus sciuri strain CD97*

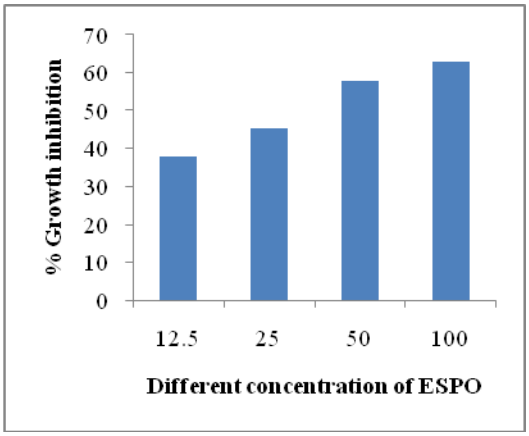


Fig14.. Percentage growth inhibition of eri silkworm pupal oil on *Staphylococcus sciuri*

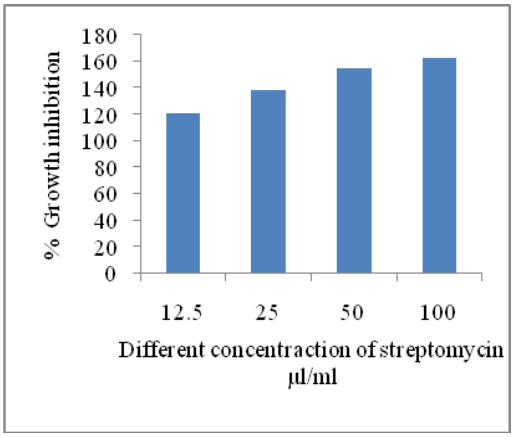


Fig.15. Percentage growth inhibition of streptomycin on *Staphylococcus sciuri strain*

2.6.8 MIC for Muga silkworm pupae oils

The tests were conducted in five test tubes with control as media with bacteria which mean±SD was 0.950±0.0001, and test sample made as media, bacteria with muga silkworm pupal oil (MUSPO) at different concentration of 12.5, 25, 50, 100µl/ml and CV%, $LSD_{0.05}$, $LSD_{0.01}$ which mean±SD values were 0.818±0.0005, 0.716±0.0055, 0.624±0.0056, 0.850±0.0361 and 0.029, 0.041, 2.088 respectively. Another five test tubes with positive control as media with bacteria which mean±SD was 0.450±0.001, and positive control made as media, bacteria and antibiotic streptomycin at different

concentration of 12.5, 25, 50 and 100µl/ml respectively which mean±SD values were 0.338±0.009, 0.220±0.011, 0.131±0.019, and 0.047±0.039 respectively showed in table 9 and figure 16, 17, 18 and 19.

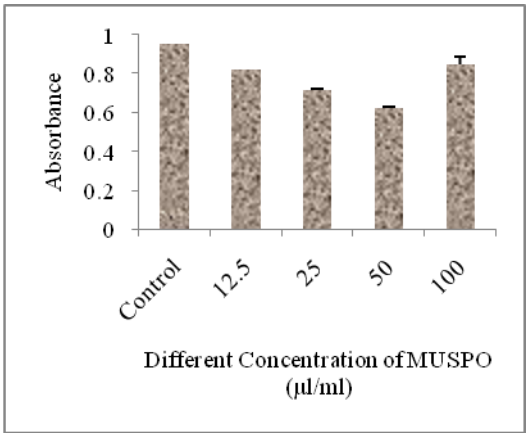


Fig.16. MIC of muga silkworm pupal oil on *Staphylococcus sciuri strain CD97*

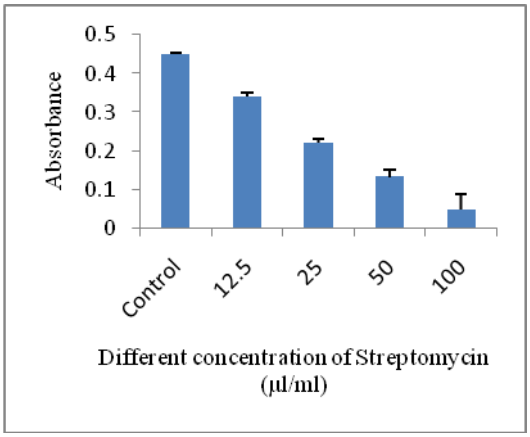


Fig.17. MIC of streptomycin on *Staphylococcus sciuri strain CD97*

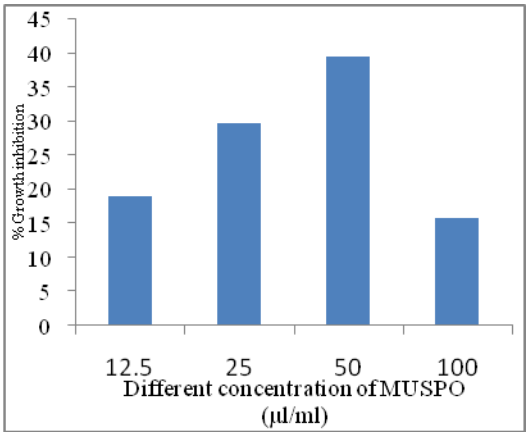


Fig.18. Percentage growth inhibition of muga silkworm pupal oil on *Staphylococcus sciuri*

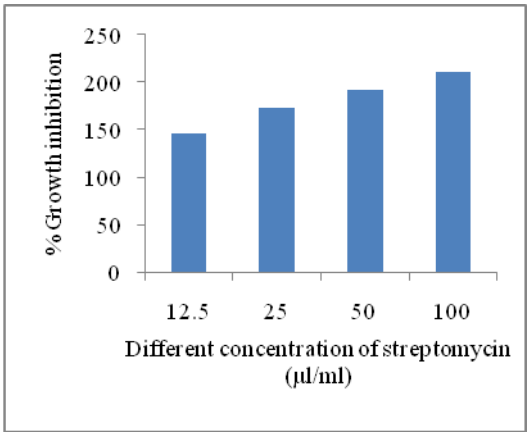


Fig.19. Percentage growth inhibition of streptomycin on *Staphylococcus sciuri*

Table: 8. Minimum inhibitory concentration of eri silkworm pupae oils, streptomycin and percentage growth inhibition on *Staphylococcus sciuri strain CD97*

Concentration (µl/ml)	Eri pupal oil Mean±SD	% Growth inhibition by Eri Pupae oils	Streptomycin Mean±SD	% Growth inhibition by Streptomycin
Control	0.556±0.0005	-	0.556±0.00057	-
12.5	0.508±0.0005	37.913	0.328±0.01050	120.671
25	0.254±0.0037	45.213	0.232±0.01594	137.926
50	0.238±0.0010	57,843	0.138±0.001	154.943
100	0.395±0.0015	62.826	0.095±0.00152	162.612

Table: 9. Minimum inhibitory concentration of muga silkworm pupae oils, streptomycin and percentage growth inhibition on *Staphylococcus sciuri strain CD97*

Concentration (µl/ml)	Muga pupal oil Mean±SD (OD)	% Growth inhibition by Muga Pupae oils	Streptomycin Mean±SD (OD)	% Growth inhibition by Streptomycin
Control	0.950±0.001		0.45086±0.001	
12.5	0.818±0.0005	19.042	0.3389±0.009	146.621
25	0.716±0.005	29.783	0.2201±0.011	172.97
50	0.624±0.005	39.49	0.13186±0.019	192.547
100	0.850±0.036	15.725	0.0478±0.039	211.193

2.7 Discussion

According to **Meetali Deori *et al.*, (2014)** the major fatty acids compounds found in muga silkworm *Antheraea assamensis* were palmitic acid (23.36%), oleic acid

(15.31%), stearic acid (5.83%), linoleic acid (2.76%) and palmitoleic acid (0.73%). The fatty acid components of the oak and mulberry silkworm pupae oils were analyzed by Gas chromatography-mass spectroscopy (GC/MS). Eight compounds were identified from the oak silkworm pupae oils, including palmitic acid, palmitoleic acid, heptadecanoic acid, stearic acid, oleic acid, linoleic acid, alpha-linolenic acid and 10 (*z*), 13 (*z*), 16 (*z*) -nonadecatrienoic acid. Palmitic acid, alpha -linolenic acid and oleic acid, as the main components in oak silkworm pupae oils showed 19.92%, 34.27% and 30.97% respectively in the area of the peaks from the GC. The heptadecanoic acid and 10 (*z*), 13 (*z*), 16 (*z*) -nonadecatrienoic acid, however, were not detected in the mulberry silkworm pupae oils. The oak silkworm pupae oils is rich in unsaturated fatty acids (77.29% of the total fatty acids) including monounsaturated fatty acids in 35.74% and polyunsaturated fatty acids in 41.55%. All of these values are higher than the value for the corresponding unsaturated fatty acid in the mulberry silkworm pupae oils (the corresponding proportions are 64.64%, 26.61% and 38.03%, respectively). The oak silkworm pupae oils contains a slightly lower proportion of alpha-linoleic acid (34.27%) than the oils from mulberry silkworm pupae (38.02%), but higher than those in soybeans and sunflowers **(Jokic., 2010)**. In our previous results, alpha-linoleic acid in spent mulberry silkworm pupae is 27.99% **(Wei *et al.*, 2009)**, which is lower than that extracted from fresh mulberry silkworm pupae (38.02%) in the present research. The above results demonstrate that the conditions of drawing silk from cocoons (e.g., high temperature, alkali, boiling water) may affect the composition of the silkworm pupae oils **(Shanker *et al*. 2006)**. Oleic acid, a monounsaturated fatty acid (MUFA), is the second-most abundant oil in oak silkworm pupae oils (30.97%). The main saturated acid in the oak silkworm

pupae oils is palmitic acid, followed by stearic acid. **Supanida Winitchai., (2011)** oils extracted from five native Thai silkworm varieties, Keaw Sakon, Nangnoi Srisaket,Sam Rong, Nang Luang and None Ruesee. The yields of the oils by the Soxhlet and maceration methods were in the ranges 24–29 and 5–7%, respectively. The silkworm pupae oil obtained from Soxhlet extraction had unsaturated fatty acid content in the range 72–79% and alpha-linolenic acid content in the range 32–44%, whereas that obtained from the maceration extraction had unsaturated fatty acid content in the range 75–80% and alpha-linolenic acid content in the range 40–46%. The study indicated that oil from native Thai silkworm pupae could be used as an alternative in the food and cosmetic industries. (**Kwon *et al.*, 2012)** studied on Isolation and analysis of natural compounds from silkworm pupae oils. The methylated fatty acid mixture was analyzed for identification of oil contents by GCMS. From this analysis he found that (Alpha-linolenic acid (omega-3) + linolenic acid) 49.0%, oleic acid 19.9%, palmitoleic acid 2.5%, stearic acid 8.6% and palmitic acid 19.7% fatty acids are present in the silkworm pupae oils. The silkworm pupae oils were found to comprise more than 40% alphalinolenic acid, (**Kwon *et al.*, 2012)**. **Thingnganing *et al.*, (2012)** studied on Eri silkworm: a source of edible oil with a high content of alpha-linolenic acid and of significant nutritional value. They also investigate fatty acid profile of eri silkworm pupae oils, pre-pupae oils (castor & tapioca), pupae oils (castor & tapioca) and *Bombxy mori* pupae oils by gas chromatography equipped with flame ionization detector (Shimadzu, Kyoto, Japan).

Silkworm pupae have a lot of potential and many applications as a natural medicine to support human health. Fatty acids composition of silkworm pupae oils were revealed by high-pressure liquid chromatography and gas chromatography mass

spectroscopy analyses and found alpha-linolenic acid {ω-3 fatty acid (antioxidant) + linoleic acid)} (49.0%), and also contain non-essential fatty acids, oleic acid (19.9%), palmitoleic acid (2.5%), palmitic acid (19.7%), stearic acid (8.6%), and eicosapentaenoic acid (EPA) (0.3%) **(Kwon *et al.*, 2012)**.

Studied on mulberry and non-mulberry silkworm pupal oil and quantified the Omega-3 (linolenic acid) fatty acids by GCMS method and found that Linolenic acid was present in mulberry, tasar, eri and muga silkworm pupae oils were 0.69%, 32.08%, 20.95% and 10.91% respectively. Whereas in my study, we found that the main fatty acid composition of the silkworm pupae oils was analyzed by gas chromatography-mass spectroscopy (GC/MS). GC-MS of mulberry and non-mulberry (tasar, eri and muga) silkworm pupae oils found that mainly four compounds in mulberry (palmitic acid C16:0 and stearic acid C18:0, linoleic acid C18:2 and linolenic acid C18:3), five in tasar (palmitic C16:0 and stearic C18:0, Oleic C18:1, linoleic C18:2 and linolenic acid C18:3), three in eri (palmitic C16:0 and stearic acid C18:0 and linolenic C18:3) and five in muga (palmitic acid C16:0 and stearic acid C18:0, linoleic acid C18:2, linolenic acid C18:3 and oleic acid C18:1.

Priyadarshni and Revansiddaiah (2013) studied on fatty acids composition in pupae oils of *Philosamia ricini* by Gas Chromatography (GC). The GC results were based on the Retention time compared with the standard fatty acids revealed that the male and female *Philosamia ricini* pupae oils exhibited twelve fatty acids among fourteen fatty acids were traced in which Linolenic acid was the major fatty acid with 46.68 % of male and 48.18% of female pupae oils during 216 hrs of development. However, the Palmitic acid (24.28%) and Oleic acid (12.21%) was found to be high in male pupae oils when

compared to female pupae oils 22.57% and 11.86% respectively. It was observed that the moderate amount of Linoleic acid and steric acid was found in both sexes. However, Caproic acid, Caprillic acid, Capric acid Lauric acid, Myristic acid, Bhenic acid and Erucic acids were found to be in traces among both sexes. Moreover, the percentage of twelve fatty acids varied significantly from 0 hrs to 216 hrs.

Liu *et al*., (2015) silkworm pupae oils derived from reeling waste is a rich source of alpha-linolenic acid (ALA), which has numerous applications. ALAs, were added in sn-1, 3 positions in triacylglycerols (TAG) to produce an APA human milk fat analog (APA-HMFAs, A: α-linolenic acid, P: palmitic acid). The most favorable situation is that tripalmitin to free fatty acids of 1:12 (mole ratio) at 65°C for 48 h using lipase Lipozyme RM IM. Results show that the most important TAG species that comprise APA-HMFAs were affluent in ALA and palmitic acid, which contain 64.52% entire unsaturated fatty acids (UFAs) and 97.05% PA at the sn-2 position. The melting point of APA was −27.5°C which is much lesser than tripalmitin (40.5°C) representing the extra plastic character. In addition, the practical application of alkyl caffeates as liposoluble antioxidants in APA was developed. Alkyl caffeate showed a superior IC_{50} (1.25–1.66μg/mL) compare to butyl hydroxy anisd (1.67μg/mL) and L-ascorbic acid-6-palmitate (L-AP) (1.87μg/mL) in DPPH analysis. The addition of ethyl caffeate to oils achieves a privileged UFAs content (73.58%) at high temperatures. Overall, APA was obtained from silkworm pupae oil successfully, and the addition of caffeates extended storage ranges for APA-HMFAs.

Priyadharshini were studied on pupae skin. Pupae skin is made up of chitin and chitosan, which is a polysaccharide and is the second most abundant material available on

earth. The chitin was evaluated in vitro for antibacterial activity against gram-negative Escherichia *coli* and gram-positive bacteria *Bacillus thuringenesis*, *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 showed 17.5 mm, 15.0 mm, 11.5 mm 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. In our studies, screened silkworm pupae oils of mulberry and non-mulberry against *Staphylococcus sciuri* strain CD97 a gram-positive bacteria and found that zone of inhibition increases by increasing the concentration of pupae oils.

The DHA rich *S. fimbriata* ***Sardinella fimbriata*** is a species of ray-finned fish in the genus *Sardinella*) extracts have an overall higher activity against all the four bacterial strains as compared to ***Sardinella*** *longiceps*. It tallies with the generalization by (**Thompson *et al.,* 1994)** that inhibitory effect of certain bacterial strain increase in the level of unsaturation. Higher activity shown by DHA-rich extract on Gram -negative bacterial strain in this study also matches with contemporary studied at DHA **Shin *et al.,* 2007**). However, PUFA extract from both species showed inhibitory activity against both Gram-positive and Gram-negative bacterial strains. This is congruent with the previous result on EPA and DHA showing activity against a range of both Gram positive and Gram negative bacteria (**Desbois *et al.,* 2008**), (**Shin *et al.,* 2006**) (**Shin *et al.,* 2007**).

However, it is noteworthy that bacterial strains that showed negative or no activity were all gram-negative.

Long chain fatty acids well known to be inhibitory on gram on gram-positive bacteria even at low concentration (**Kabara *et al.*, 1972**). However, gram-negative bacteria are known for their complex lipopolysccharide layer as compared to the former. But PUFA is known to have an inhibitory effect on these strains as compared to saturated fatty acids as they are readily incorporated into the outer cell membranes of these organisms, where they significantly increase membrane fluidity. It is possible that by opening up permeability channels, the concentration gradient necessary between the organism and its environment may be dissipated resulting in the fatality of the organism.

Shin *et al.*, 2006 demonstrated that EPA can reduce the viability of *P. aeruginosa*. In their experiment scanning electron microscopy (SEM) study of bacterial cell clearly exhibited the antibacterial effect of EPA evidenced by the damages found in the outer membrane of the cells when treated with EPA. **Shin *et al.*, (2007)** later found out that DHA is even more potent against this bacterium. The high positive result in this in this study of inhibiting the culture of *Pseudomonas aeruginosa* could also be due to high DHA and EPA concentrated in both the extract. Higher DHA concentration of *Sardinella fimbriata* correlates with the greater inhibitory effect on this bacterium.

The fatty acids as the primary constituents in edible oils have been reported to hold the capability to hinder with bacterial growth or survival (**Mckellar *et al.*, 1992, Isaacs *et al.*, 1995, Kankaanppa *et al.*, 2001, Sprong *et al.*, 2002, Benkendroffk *et al.*, 2005, Knapp *et al.*, 1986)**. Antimicrobial activity of fatty acids was confirmed to be dependent on chain length and unsaturation level **(Benkendroffk *et al.*, 2005, Knapp *et***

***al.*, 1986)**. Long-chain unsaturated fatty acids show inhibitory action against several bacteria, even including *methicillin-resistant Staphylococcus aureus* (MRSA) **(Farrington *et al.*, 1992)**. For example, linoleic and oleic acids were reported as powerful antibacterials activity (**Dilika *et al.*, 2000, Zheng *et al.*, 2005)**. In a recent study on 3 hazelnut cultivars, oleic acid was found to range between 80.67% and 82.63%, which is similar to our hazelnut oil, and the hazelnut extract was also reported to display strong antibacterial activity against gram-positive bacteria (**Olivera I *et al.*, 2008.** In addition, tocopherol and derivatives found in walnut were previously found to produce strong antioxidant activity (**Mambro *et al.*, 2003)**. Therefore, trans-fatty acids and tocopherol derivatives widely found in the oils could be possibly responsible for the antioxidant activity of these oils. In conclusion, this study underlines that the nuts examined herein are not only sources of energy, but also provides important components, such as monounsaturated and polyunsaturated fatty acids with antioxidant and antimicrobial activities.

Linum usitatissimum fixed oil showed good in-vitro antimicrobial activity against a number of microorganisms including *Staphylococcus aureu*, *Staphylococcus agalactiae, Enterococcus faecalis,* which are commonly associated with mastitis **(Kaithwas *et al.*, 2010)**. In our study the mulberry and non-mulberry silkworm pupal oils showed antimicrobial activity against *Staphylococcus sciuri* strain CD_{97} a gram-positive bacteria.

Mulberry and non mulberry (tasar, eri, muga) silkworm pupae oil is an interesting sub-product obtained after the extraction procedure of silk threads. The percentage yields of mulberry, tasar, eri and muga pupal oils from the maceration methods were 2.0%,

1.2%, 2.4%, and 1.6% respectively. Physicochemical properties of Eri pupae oil studied by Sharma & Ganguly and found the viscosity 33.6%, Iodine value 128%, Saponification number, 218%.

Thumu Ravinder *et al* (2016) also studied physico-chemical properties of eri silkworm pupal oil and found the density, viscosity, iodine value, saponifiable value and unsaponifiable matter were 0.9126±0.10, 32.21±0.14, 130.90±0.31, 196.45±0.28, 3.33±0.41 respectively. While physico-chemical properties in case of mulberry (density 0.742±0.0068, viscosity 32.5±1.053, iodine value 132.08±1.799, saponifiable value 205.66±1.527 unsaponifiable matter 0.164±0.0005), tasar (density 0.884±0.01, viscosity 35.86±1.28, iodine value 133.85±2.504, saponifiable value 205±2.00 unsaponifiable matter 0.843±0.001) eri (density 0.676±0.002, viscosity 33.56±2.35, iodine value 133.88±2.019, saponifiable value 205±2.645unsaponifiable matter 0.125±0.0015) and muga (density 0.957±0.017, viscosity 34.62±0.673, iodine value 133.56±1.659, saponifiable value 206.66±1.527 unsaponifiable matter 0.883±0.1011) silkworm pupal oil respectively.

2.8 Conclusion

The omega-3 compound as alpha-linolenic acid, which is essential for the human body is present in mulberry and non-mulberry silkworm pupae oils. The alpha-linolenic compound breaks into EPA and DHA during metabolic activity. It is reported that the EPA has antimicrobial property. So, mulberry and non-mulberry silkworm pupae oils were shown antibacterial activity on *Staphylococcus sciuri* strain CD_{97}. While same pupae oil did not show any effect on the *Phyllactinia corylea* fungal strain.

CHAPTER III

ANTIOXIDANT PROPERTIES OF MULBERRY AND NON MULBERRY SILKWORM PUPAE OILS

3. Introduction

3.1 Background of the study

Mulberry has become one of the most accepted herbal medicines of modern years, even though its use dates back many hundreds of years to earliest Chinese medicine. Polyphenols such as scopoletin, querectin, morin, hydroxycoumarin, rutin, isoquercetin, querectin 3-malynoglucoside (Q3MG) had been identified as natural antioxidants in mulberry leaves. Mulberry leaf extract which contains rutin, quercetin, isoquercetin and other flavonoids, have been shown to inhibit oxidative modification of low density lipoprotein and may reduce atherosclerosis **(Katsube *et al.,* 2006).** Quercetin has been reported to demonstrate biological effects such as antioxidant. At concentrations of 75-100 m mol, morin inhibits oxidation of low density lipoprotein (LDL) by free radicals or Cu^{2+}. Scopoletin is reported to possess anti-inflammatory, immunomodulatory and antioxidant activity **Manuele *et al.,* 2006)**.

3.2 Antioxidant in Pupae

Silkworm pupae have a lot of potential and profuse applications as a natural medicine to support in human health. Silkworm pupae have antioxidants, quercetin diglucoside and it is nutritionally significant with vitamin B_2 **(Kwon *et al.,* 2012)**. The antioxidant activity of Muga silkworm pupae evaluated by **Meetali *et al.,* 2014**, therefore it is suggested that pupae can be utilized as natural antioxidants in various food products. The composition of silkworm pupae oil is analyzed by the analytical methods such as high pressure liquid chromatography and gas chromatography-mass spectroscopy and found alpha-Linolenic acid (ALA) which is 49% of fatty acid, which showed antioxidant activity and also containing non-essential fatty acids which are oleic acid (19.9%),

palmitoleic acid (2.5%), palmitic acid (19.7%), stearic acid (8.6%), and eicosapentaenoic acid (0.3%) **(Kwon *et al.,* 2012)**. Pupae oil is extracted from five native Thai silkworm varieties by using soxhlet extraction method which displayed free radical scavenging activity **(Winitchai *et al.,* 2011)**. The antioxidant activity of pupae of Muga and Eri silkworm is concluded by who found that, the pupae could be used as natural antioxidants in food products.

3.3 Antioxidant in Silkworm pupae oils

The silkworm pupae oil contains a mixture of essential fatty acids with bioactivity that consequently can be used as a raw material for cosmetics. Pupae oil is also used in the cosmetic industries for the manufacturing of soaps and moisturizers (**Kotake-Nara *et al.,* 2002**). It demonstrated that glutamic acid (18.3%), histidine (14.6%) and alanine (10.2%) are the most common amino acids present in silkworm pupae.

Silkworm pupae oil is used because of anti-oxidative property which prevents aging, caused by many oxidants. Tyrosinase inhibition activity is displayed by None Ruesee silkworm pupae oil, which determines the inhibition of melanin formulation in skin. Tyrosinase is a copper containing monooxyginase enzyme, which can be found in fungi, higher plants and animals. Moreover, it is known to be a key enzyme in melanin biosynthesis (**Leibovitz and Siegel, 1980, Gutierrez *et al.,* 2006).**

Oxidative alteration of lipids and small cellular molecules by reactive oxygen species (ROS) along with an impaired antioxidant mechanism play several roles in an extensive range of common diseases and age-related degenerative conditions. Oxidant damage by ROS is also associated with photo ageing radiation toxicity, cataract formation and muscular degeneration **(Kolawole *et al.,* 2014)**. Once free radicals are

initiated, they can propagate by involving in chain reactions with other less reactive types, the resulting chain reaction compounds generally survive longer in the body and thus increase the potential for cellular damage. To protect molecules against toxic free radicals and other ROS, cells have developed an antioxidant defense system that includes the enzymes, super oxide dismutase (SOD), which dismutates superoxide; catalase (CAT); glutathione reductase and glutathione peroxidase, which destroy toxic peroxides and small molecules including glutathione **(Cheng *et al.,* 2014).** The rise in catalase activity during larval development was directly related to the formation of pro-oxidant in the larva and checks the deleterious effect of aging through its antioxidant effect **(Amritha *et al.,* 2014)**. Dietary antioxidant have been demonstrated to be protective through the activation of hermetic pathways, including vitagenes and proteosomal activity degrading oxidetively modified proteins **(Izabela *et al.,* 2014)**. In addition to this there is an inverse relationship between dietary intake of antioxidant rich foods and the incidence of a number of diseases. Therefore search into the determination of antioxidant capacity of different compounds become important. So, the present study to assessment of physico-chemical and antioxidant properties of Mulberry and Non-Mulberry (Tasar, Eri and Muga) silkworm pupae oils.

3.4 Materials and Methods

3.4.1 Determination of physical and chemical characteristics of silkworm pupae oils

The oils were evaluated its fatty acid composition by gas liquid Chromatography using the methyl estrification method. The oil thus obtained through different extraction procedure will be evaluation of its density, viscosity, acid value, iodine value, ester value, saponification value and unsaponifiable matter.

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. Thin-layer chromatography is performed on a sheet of aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide.

3.4.2 Gas liquid chromatography of mulberry and non-mulberry silkworm pupae oils

3.4.2.1 A Methylation of non-volatile to volatility of mulberry and non-mulberry silkworm pupae oils

Samples analyzed by GC must be volatile (have a significant **vapour pressure** below 250 °C). **Derivatization** to increase volatility is possible but can be cumbersome and introduces possible **quantitative** errors. Most GC analytes are under 500 Da Molecular Weight for volatility purposes. Highly polar analytes may be less volatile than suspected when dissolved in a polar solvent or in the presence of other polar species due to intermolecular forces such as hydrogen bonding.

So, before GLC analysis a methylation process has been done both mulberry and non-mulberry silkworm pupal oils with following method in CIMAP (Central Institute of Medicinal and Aromatic Plants) Lucknow Uttar Pradesh India. After extraction of mulberry, tasar, eri and muga silkworm pupal oil from their pupae were in non-volatile form. In order to analyze the above mentioned oils with GLC, the volatile nature of all varieties of oil was converted into volatile form with the following the procedure described by **Hammond (1993)**. Briefly, 50 mg of petroleum ether extract of each variety of silkworm pupal oil were dissolved in 5ml of reagent made up of

H_2SO_4/MeOH/Toluene (1:10:20, v/v, toluene used as solubilizer) and refluxed for 1 hour. The reaction mixture was ready for GC/MS study.

3.4.3 Density of mulberry and non mulberry silkworm pupae oil

To find out the density of silkworm pupal oil.

$$\text{Density} = \frac{\text{Mass}}{\text{Volume}}$$

3.4.3.1 Chemical and apparatus

2 ml syringe, weight balance.

3.4.3.2 Procedure

Find the weight of empty 2 ml syringe, then syringe was filled with mulberry silkworm pupal oil and weights it.

The weight of silkworm pupal oil filled in syringe = weight of syringes filled with pupal oil- weight of empty syringe

So, Density=mass/volume

3.4.4 Viscosity of mulberry and non mulberry silkworm pupae oils

3.4.4.1 Materials required

A stopwatch, a tall graduated cylinder, steel ball, silkworm pupal oil

3.4.4.2 Procedure

Filled two graduated cylinder with two different liquids. Drop 1 steel ball into the first liquid. Repeated two more times. Used a stopwatch to record how long it takes the steel ball to reach the bottom. Recorded results data time it takes for a steel ball to sink through a liquid.

3.4.4.3 Chemical and apparatus

Siphoning pipe, Test tube stand, Match box, Candle.

3.4.4.4 Procedure

Sealed the plastic siphoning tube at one end by heating and stand with the help of test tube stand. Then it filled by silkworm pupal oil, and after filling put a small steel boll into test tube and calculates the time of ball upto reached the bottom.

3.4.5 Acid value of mulberry and non mulberry silkworm pupae oils

The acid value (AV) is a number which express the quantity of potassium

3.4.5.1 Materials required

Silkworm pupal oil, Absolute ethanol alcohol, Phenolphthalein, 0.1 N KOH.

3.4.5.2 Procedure

5.0 gm of silkworm pupal oil was taken in a dried conical flask. Added 25 ml of absolute ethanol alcohol and add (2-3) drops of phenolphthalein. Heat with shaking in water bath (65%) for 10 minutes, then cool it and titrate the solution against 0.1 N KOH until pink color appears (end point). Observations were recorded as:

Calculated the acid value (AV) by using formula:

$$AV = \frac{\text{ml of KOH x N x 56}}{\text{Weight of Sample}} = \text{mg of KOH}$$

3.4.6 Iodine value of mulberry and non mulberry silkworm pupae oils

One of the most commonly used methods for determining the iodine value of lipids is "Hanus method". The lipid to be analyzed is weighed and dissolved in a suitable

organic solvent, to which a known excess of iodine chloride is added. Some of the I Br reacts with the double bonds in the unsaturated lipids, while the rest remains:

$$R\text{-}CH{=}CH\text{-}R + I\,Br_{excess} ======== R\text{-}CHI\text{-}CH\,Br\text{-}R + I\,Br_{remaining}$$

The amount of I Br that has reacted is determined by measuring the amount of I Br remaining after the reaction has gone to completion ($I\,Br_{reacted} = I\,Br_{excess} - I\,Br_{remaining}$). The amount of I Br remaining is determined by adding excess potassium iodide to the solution to liberate iodine, and then titrating with a sodium thiosulfate ($Na_2S_2O_3$) solution in the presence of starch to determine the concentration of iodine released:

$$I\,Br_{remaining} + 2KI ========= KBr + KI + I_2$$

$$I_2 + \text{starch} + 2Na_2S_2O_3\ (\text{blue}) ====== 2NaI + \text{starch} + Na_2S_4O_6\ (\text{colorless})$$

3.4.6.1 Materials

Mulberry, tasar, eri and muga silkworm pupae oils. Hanus solution (it's prepared by dissolving 18.2 g of iodine in 1L of glacial acetic acid and then add 3 ml of bromine water for increasing the halogen content. 15% potassium iodide solution. 1% starch solution. 0.1 N Sodium thiosulfate solution.

3.4.6.2 Procedure

Taken 0.25 g of pupae oil into a 250 mL conical flask and added 10 ml of chloroform, 30 ml of Hanus solution and close the flask completely by Para film, then leave the solution for 30 minutes with shaking continuously on shaker. Then added 10 ml of 15% potassium iodide solution and then shake. Added 100 ml of distilled water (DW). Now titrated the iodine solution against 0.1 N Sodium thiosulfate solution till yellow

color formed, then added 2-3 drops of starch solution where blue solution formed and then continue with titration till the blue color is disappeared (Volume (ml) of $Na_2S_2O_3$ at endpoint represents S)

1. Did the same above procedure, but without a sample (Volume (ml) of $Na_2S_2O_3$ at endpoint represents B).
2. Calculated the iodine number by using the following law:

$$\text{Iodine Value} = \frac{(B - S) \times N \text{ of } Na_2S_2O_3 \times 0.127 \text{g/meq} \times 100}{\text{Weight of Sample (g)}}$$

B: V ml of $Na_2S_2O_3$ volume for blank

S: V ml of $Na_2S_2O_3$ volume for sample

3.4.7 Ester value of mulberry and non mulberry silkworm pupae oils

It is defined as the mg of KOH required to react with glycerin after saponify one gram of fat. It is calculated from the saponification value (SV) and the acid value (AV):

Ester Value (EV) = Saponification Value (SV) – Acid Value (AV)

3.4.8 Saponification value of mulberry and non mulberry silkworm pupae oils

3.4.8.1 Materials required

Silkworm pupal oil, 0.5N methanolic KOH, 0.5N HCl, Phenolphthalein indicator, Buratte, conical flask, micropipette, funnel.

3.4.8.2 Methods

Taken 5 g of pupae oil in a conical flask added 50 ml of 0.5 M methanolic KOH in it Kept the flask on a boiling water bath and boiled until oil is completely saponify (30 minutes approx) cool and titrate it with 0.5 M HCl using phenolphthalein indicator. Conduct the blank determination along with the sample, using same period for Formula:

$$\text{Saponification value} = \frac{\text{Concentration conversion factor (28.05)} \times (\text{Blank} - \text{Sample})}{\text{Weight of the sample taken}}$$

3.4.9 Unsaponifiable matter of mulberry and non mulberry silkworm pupae oils

3.4.9.1 Principle Reagent

All reagents shall be of analytical reagent quality. Ethyl alcohol, 95% ethyl ether, free from peroxides, acetone concentrated aqueous potassium hydroxide solution, approximately 50% KOH by weight. Prepared by dissolving 60g KOH in 40 ml of water and cooling. Aqueous potassium hydroxide solution; about 0.5 N, prepared by dissolving 30g KOH in water, cooling and diluting to 1 liter. Aqueous sodium hydroxide solution; 0.02N accurately standardized. Phenolphthalein indicator solution, 1% in 95% alcohol.

3.4.9.2 Apparatus

Separating funnels, 250ml capacity Erlenmeyer flasks, narrow mouth, 200ml with 20/40 standard taper outer joint. Condensers, with 20/40 standard taper joint to fit the

200ml Erlenmeyer flasks; Air condensers may be used. Beakers 250ml. Erlenmeyer or flat bottom fat extraction flasks, 50ml. Glass stoppered cylinders, about 300mm height and 35mm diameter to contain at least 150ml. Glass siphon.

3.4.9.3 Method

Weigh accurately 2.5 g of well mixed silkworm pupal oil into the 200 ml Erlenmeyer flask. Added 25 ml of alcohol and 1.5 ml of concentrated aqueous KOH solution (50%) and mix. Boil gently, with occasional swirling, under a reflux condenser for 30 minutes. No loss of alcohol should occur during saponification. Transfer while warm to the extraction cylinder, washing with a total of 50 ml of water. Wash the flak with 50 ml of ethyl ether and add to the cylinder. Cool contents to room temperature (20˚C to 25˚C). Insert the stopper and shake vigorously, taking usual precautions to release any pressure that may develop in the cylinder. Allow the layers to separate and clarify. Use a glass siphon to remove the upper layer as completely as possible and transfer the ether to a 250 ml separating funnel containg 20ml of water.

Repeat the extraction two more time with 50 ml portions of ethyl ether, shaking vigorously. Adjust the siphon after each separation in order to remove the ether layer as completely as possible.

Rotate the combined ether extracts gently with the 20 ml of water. Violent agitation at this stage may result in troublesome emulsions. Allow the layers to separate completely and draw off the lower aqueous layer. Wash the ether twice more with the 20 ml portions of water, shaking vigorously each time and discarding the lower aqueous layer after separation. Wash the ethyl ether solution three times with 20 ml portions of

about 0.5 N aqueous KOH, shaking vigorously each time, and fallow each alkali treatment by washing with 20 ml of water. If an emulsion form during this washing procedure, allow separating as much as possible, discard the clear aqueous layer, and proceed with the next step, leaving any emulsion in the separating funnel with the ether layer. After the third washing with the 0.5N KOH, wash the other with successive 20 ml portions of water until the washing is no longer alkaline to phenolphthalein. Transfer the ether solution to a 250 ml beaker, raising the separating funnel and its pouring edge with ether and adding the rinsing to the main solution. Evaporate to about 5 ml and transfer quantitatively with the aid of several small portions of ether to a 50 ml of fat extraction flask which has been previously dried and weighed. Place flak on a steam bath to remove the ether. When practically all of the ethers evaporated add 2 or 3 ml of acetone and remove all solvent completely by passing a gentle current of clean dry air through the warmed flask. Complete the drying to constant weight, preferable in a vacuum oven at 75˚C to 80˚C and an internal pressure of not more than 200 mm of mercury. Cool in desiccators and weigh. After weighing dissolve contents of flask in 2 ml of ethyl ether and then add 10 ml of 95% alcohol previously neutralized to a faint pink colour using phenolphthalein indicator. Titrate with 0.02N NaOH to the same final colour. Correct weight for residue for free fatty acid content (1ml of 0.02N NaOH is equivalent to 0.0056g of oleic acid). Also correct the weight of the residue for reagent blank obtained by conducting the determination in the same manner but omitting the oil or fat.

3.4.9.4 Calculation

Unsaponifiable matter % =

$$\frac{\text{(wt. of residue – wt. of fatty acid – wt. of blank) X 100}}{\text{Wt. of sample}}$$

After completion of physico-chemical analysis of mulberry and non-mulberry silkworm pupal oils further analysis of antioxidant activity of mulberry, tasar, eri and muga silkworm pupal oils.

3.4.10 In vitro antioxidant activity of mulberry and Non-mulberry silkworm pupal oils by using following methods:

3.4.11 DPPH free radical scavenging activity

3.4.11.1 Preparation of standard solution

Required quantity of Ascorbic acid was dissolved in methanol to give the concentration of 5, 10, 20, 30, 40 and 50µg/ml.

3.4.11.2 Preparation of test sample

Stock solutions of samples were prepared by dissolving 10 mg of mulberry and non-mulberry silkworm pupal oil in 10 ml of methanol to give concentration of 1mg/ml.

3.4.11.3 Preparation of DPPH solution

4.3mg of DPPH was dissolved in 3.3 ml methanol: it was protected from light by covering the test tubes with aluminum foil.

3.4.11.4 Protocol for estimation of DPPH scavenging activity

150µl DPPH solution was added to 3 ml methanol and absorbance was taken immediately at 516 nm for control reading. Different volume levels of test sample (10 µl,

20 µl, 30 µl, 40 µl and 50 µl) were screened and made 200 µl of each dose level by dilution with methanol. Diluted with methanol with up to 3 ml. 150µl DPPH solution was added to each test tube. The reaction was allowed to take place in the dark at room temperature. The absorbance at 516 nm was measured at different time intervals. A decreasing intensity of the purple colour was taken as increasing scavenging activity. Ascorbic acid served as a standard. After 15 min using methanol as a blank. The % reduction and IC_{50} were calculated as follows. (**Sanja *et al.*, 2009**)

The free radical scavenging activity (FRSA) (% antiradical activity) was calculated using the following equation:

3.4.11.5 Inhibition (%) = $[(A_0-A)/A_0] \times 100$

Where A_0 is the absorbance of DPPH in the absence of the sample and A is the absorbance of DPPH in the presence of sample.

Each experiment was carried out in triplicate and results are expressed as mean % antiradical activity ± SD.

3.4.12 FRAP assay method

The ferric reducing powers of the mulberry and non-mulberry silkworm pupal oils were determined by using the potassium ferricyanide ferric chloride method. Different concentrations of mulberry and non-mulberry silkworm pupal oil (10 µl, 20 µl, 30 µl, 40 µl, 50 µl /ml) were added to 2.5 mL phosphate buffer (0.2M, pH 6.6) and 2.5 mL potassium ferricyanide (1%). The mixtures were incubated at 50°C for 20 min, after which 2.5 mL trichloroacetic acid (10%) was added. An aliquot of the mixture (2.5 mL) was taken and mixed with 2.5 mL absolute alcohol and 0.5 mL 1% $FeCl_3$. The absorbance at 700 nm was measured after allowing the solution to stand for 30 min. The

FRAP of a sample is estimated in terms of Trolox equivalent antioxidant capacity (TEAC) in mM/L Trolox. Each assay was carried out in triplicate. Higher absorbance of the reaction mixture indicated greater reducing power.

3.4.13 Hydrogen peroxide assay

Hydrogen peroxide-scavenging activity The Hydrogen peroxide-scavenging activity of silkworm pupal oil was determined by the method of **Ruch *et al.*, (1989**). The different concentration 10 µl, 20 µl, 30 µl, 40 µl, 50 µl /ml pupal oil were dissolved in 3.4 mL of 0.1M phosphate buffer (pH 7.4) and mixed with 600 µL of 43 mM solution of hydrogen peroxide . The absorbance value (at 230 nm) of the reaction mixture was recorded at 10 min intervals between zero and 40 min. for each concentration, a separate blank sample was used for background substraction. The absorbance at 516 nm was measured at different time intervals. The experiment was performed in triplicate and percentage inhibition was calculated using the formula.

3.4.13.1 Inhibition (%) = $[(A_0-A)/A_0] \times 100$

Where A_0 is the absorbance of control in the absence of the sample and A is the absorbance in the presence of sample.

3.4.14 Super oxide scavenging assay

3.4.14.1 Chemicals and Reagents

Dimethyl sulfoxide was purchased from Biotech park, Nitro-blue Tetrazolium was purchased from CDRI Lucknow.

3.4.14.2 Preparation of standard solution

10 mg of ascorbic acid dissolved in 10 ml of absolute alcohol. Dilutions of this solution with absolute alcohol were prepared to give the concentration of 10 µl, 20 µl, 30 µl, 40 µl and 50 µl / ml.

3.4.14.3 Preparation of test sample

Dissolve 25 ml of mulberry and non-mulberry silkworm pupal oil in 25ml of dimethyl sulfoxide to give stock solution of 1 ml/ml. Dilution were done with same dimethyl sulfoxide to give concentrations of 10 µl, 20 µl, 30 µl, 40 µl, 50µl/ml.

3.4.14.4 Preparation of reagents

Alkaline DMSO: 1 ml alkaline DMSO containing, 5 mM NaOH in 0.1 mL absolute alcohol and 0.9 ml Dimethyl sulfoxide. NBT: 25 mg of nitro-blue Tetrazolium was dissolved in 25 ml of Dimehtyl sulfoxide to give concentration of 1 mg/ml.

3.4.14.5 Protocol for estimation of superoxide scavenging activity

To the reaction mixture containing 0.1 ml of NBT (1 mg/mL solution in DMSO) and 0.3 ml of the pupal oil and standard in DMSO, 1 ml of alkaline DMSO (1 mL DMSO containing, 5 m M NaOH in 0.1 ml alcohol) was added to give a final volume of 1.4 ml and the absorbance was measured at 560 nm. Pupal oil (100-600 µl/ml) was added to a hydrogen peroxide solution (0.6ml, 40mM). 300 µl of plain DMSO, 0.1 ml NBT solution and 1 ml alkaline DMSO was mixed and absorbance was taken at 560 nm and this was taken as control reading. The percentage of super oxide radical scavenging by the mulberry and non-mulberry silkworm pupal oil and standard compounds were calculated as follows:

$$\% \text{ super oxide scavenging activity} = \frac{\text{test absorbance} - \text{control absorbance} \times 100}{\text{Test absorbance}}$$

3.4.15 Reducing power of mulberry and non-mulberry silkworm pupal oil

3.4.15.1 Preparation of standard solution

10mg of ascorbic acid dissolved in 10 ml of absolute alcohol. Dilutions of this solution with absolute alcohol were prepared to give the concentration of 10 µl, 20 µl, 30 µl, 40 µl and 50 µl/ml.

3.4.15.2 Preparation of test sample

Required quantities of mulberry and non-mulberry silkworm pupal oil were dissolved in minimum quantity of alcohol and volumes were made up to 10 ml with phosphate buffer. Separately all the samples were diluted in 10 ml volumetric flask with phosphate buffer to give 10 µl, 20 µl, 30 µl, 40µl and 50µl/ml concentration.

3.4.15.3 Preparation of reagents

Phosphate buffer: 0.2M phosphate buffer of ph 6.6 was prepared according to I.P. 1% Potassium ferricyanide solution: 2 gms of potassium ferricyanide was dissolved in 200ml of absolute alcohol. 10% Trichloro acetic acid: 40 gms of Trichloro acetic acid was dissolved in 400ml of absolute alcohol. 0.1% ferric chloride solution: 0.1 gm of ferric chloride was dissolved in 100ml of absolute alcohol.

3.4.15.4 Protocol for reducing power

2ml of each sample and standard solutions were spiked with 2.5ml of 1% Potassium ferricyanide solution. This mixture was kept at 50º C in water bath for 20 min. After cooling, 2.5 ml of 10% Trichloro acetic acid was added and centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml of absolute alcohol and 1 ml of 0.1% ferric chloride and kept for 10 min. Control was prepared in similar manner excluding samples. The absorbance of resulting solution was measured at 700 nm.

3.5 **Results**:

3.5.1 Fatty acids in mulberry and non mulberry silkworm pupae oils

The fatty acid components present in silkworm pupae oil were analyzed by gas chromatography mass spectroscopy (GC/MS). Five compounds were identified from the silkworm pupae oil, including alpha linolenic acid, Linoleic acid, oleic acid, palmitic acid, and stearic acid,

The fatty acid components of the silkworm pupae were analyzed by gas chromatography-mass spectroscopy (GC/MS). Five compounds were identified from the silkworm pupae, including oleic acid, palmitic acid, stearic acid, linoleic acid, and palmitoleic acid. Palmitic acid and oleic acid, as the main components in the pupae showed 23.36% and15.31% respectively in the area of the peaks from GC. The muga silkworm pupae is rich in saturated and unsaturated fatty acid comprising of palmitic acid and stearic acid respectively and oleic acid, linoleic acid, and palmitic acid respectively. These two types of fatty acid are essential for human body. The human body cannot produce this linoleic acid, so human bodies get it from food (**Sappayatosok, 1988**). Some researcher reported that the neutral lipid of silkworm pupae of *Bombyx mori* was considered to be a good source of alpha-linolenic acid (ALA). Conjugated linoleic acid isomers have been reported to have biological activity. Dietary conjugated linoleic acid reduced tumorigenesis and metastasisofbreasts, prostrate, skin and colon cancer in experimental animals.

Pic 11. TLC analysis of mulberry and non mulberry silkworm pupal oil to after methylation

The abbreviation coted on TLC plate were given below:

A= Pure mulberry silkworm pupal oil

A1= Methylated mulberry silkworm pupal oil

B= Pure tasar silkworm pupal oil

B1= Methylated tasar silkworm pupal oil

C= Pure eri silkworm pupal oil

C1= Methylated eri silkworm pupal oil

D= Pure muga silkworm pupal oil

D1= Methylated muga silkworm pupal oil

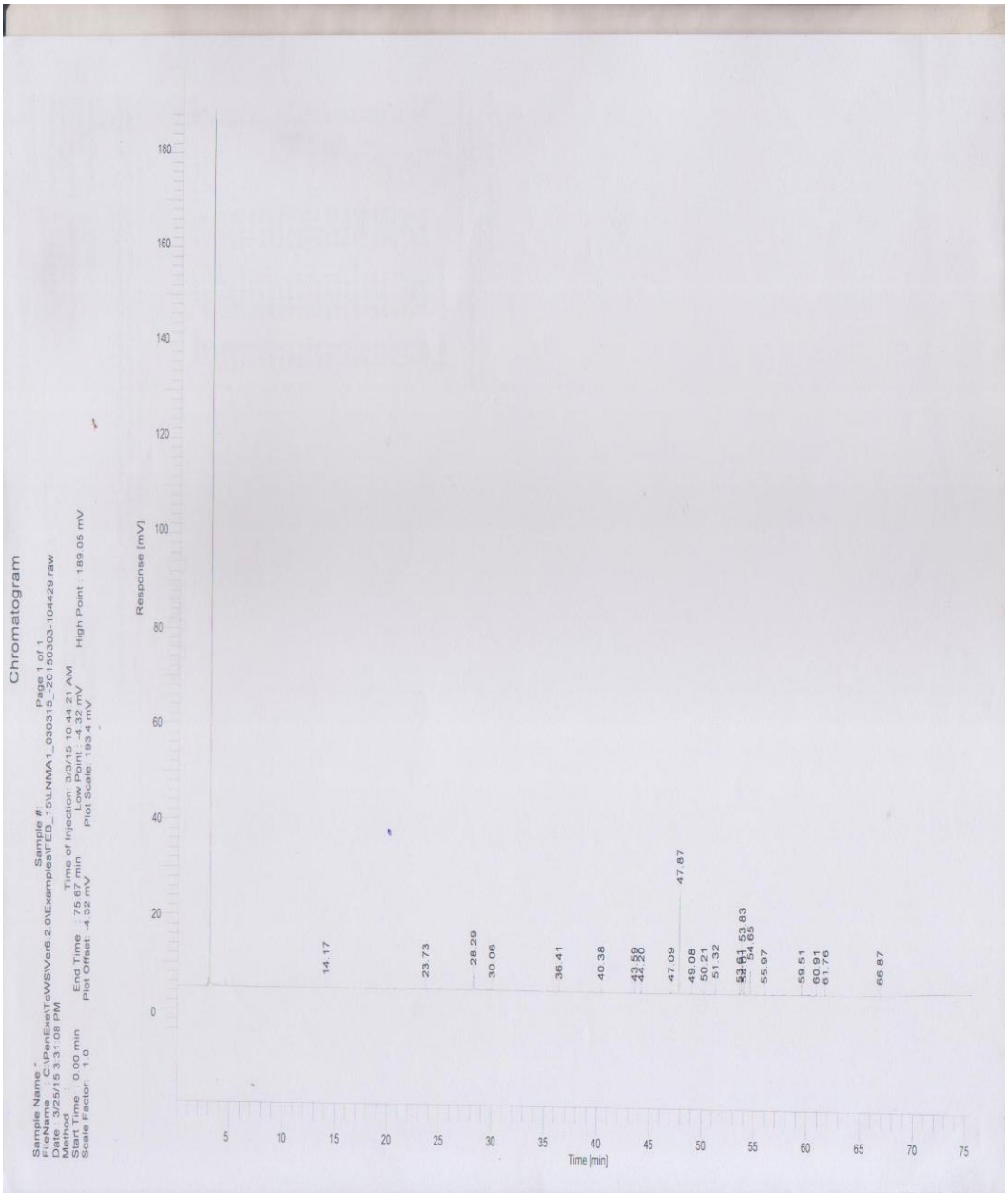
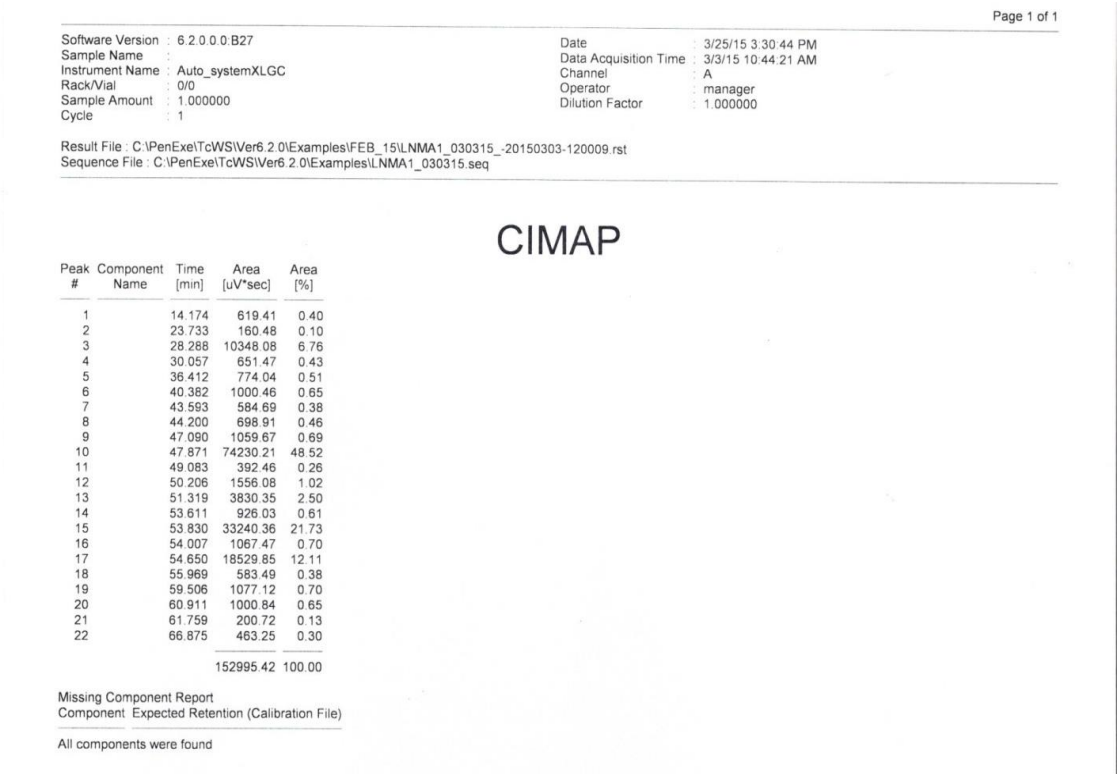


Fig.20. GCMS of mulberry silkworm pupal oil



Page 1 of 1

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

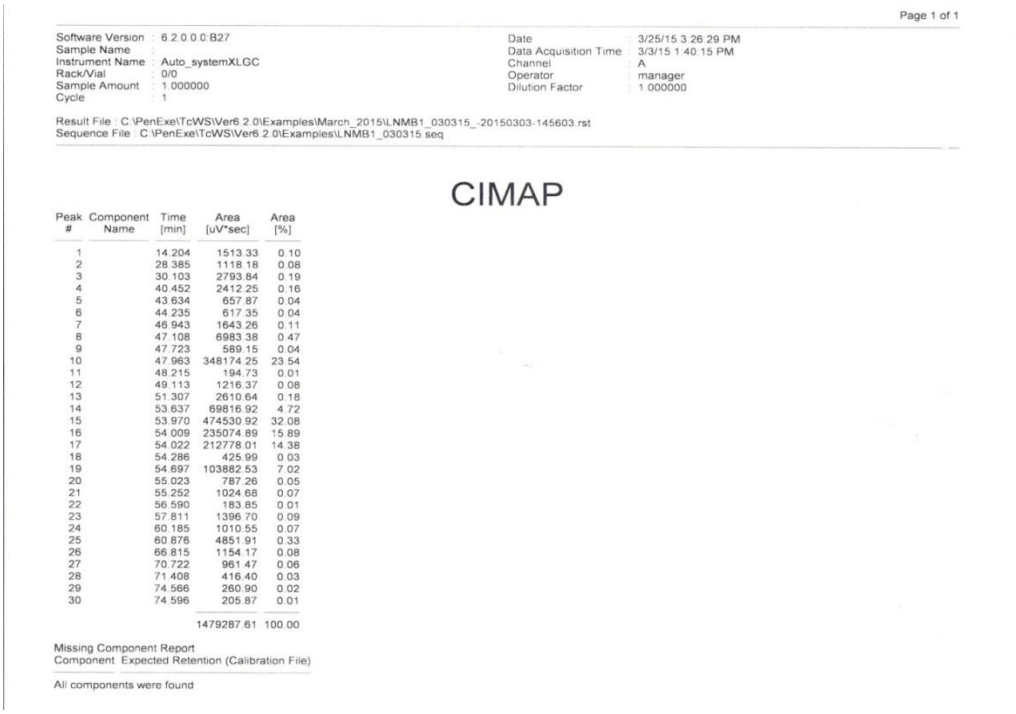
CIMAP

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	1059.67	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	1067.47	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 components were found

Fig.21. Retention time of mulberry silkworm pupae oils



Page 1 of 1

Software Version : 6.2.0.0.0:B27
Sample Name :
Instrument Name : Auto_systemXLGC
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Sample Amount : 1.000000
Cycle : 1

Date : 3/25/15 3:26:29 PM
Data Acquisition Time : 3/3/15 1:40:15 PM
Channel : A
Operator : manager
Dilution Factor : 1.000000

Result File : C:\PenExe\TcWS\Ver6.2.0\Examples\March_2015\LNMB1_030315_-20150303-145603.rst
Sequence File : C:\PenExe\TcWS\Ver6.2.0\Examples\LNMB1_030315.seq

CIMAP

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
1		14.204	1513.33	0.10
2		28.385	1118.18	0.08
3		30.103	2793.84	0.19
4		40.452	2412.25	0.16
5		43.634	657.87	0.04
6		44.235	617.35	0.04
7		46.943	1643.26	0.11
8		47.108	6983.38	0.47
9		47.723	589.15	0.04
10		47.963	348174.25	23.54
11		48.215	194.73	0.01
12		49.113	1216.37	0.08
13		51.307	2610.64	0.18
14		53.637	69816.92	4.72
15		53.970	474530.92	32.08
16		54.009	235074.89	15.89
17		54.022	212778.01	14.38
18		54.286	425.99	0.03
19		54.697	103882.53	7.02
20		55.023	787.26	0.05
21		55.252	1024.68	0.07
22		56.590	183.85	0.01
23		57.811	1396.70	0.09
24		60.185	1010.55	0.07
25		60.876	4851.91	0.33
26		66.815	1154.17	0.08
27		70.722	961.47	0.06
28		71.408	416.40	0.03
29		74.566	260.90	0.02
30		74.596	205.87	0.01
			1479287.61	100.00

Missing Component Report
Component Expected Retention (Calibration File)

All components were found

- **Fig.22. Retention time of tasar silkworm pupal oil**

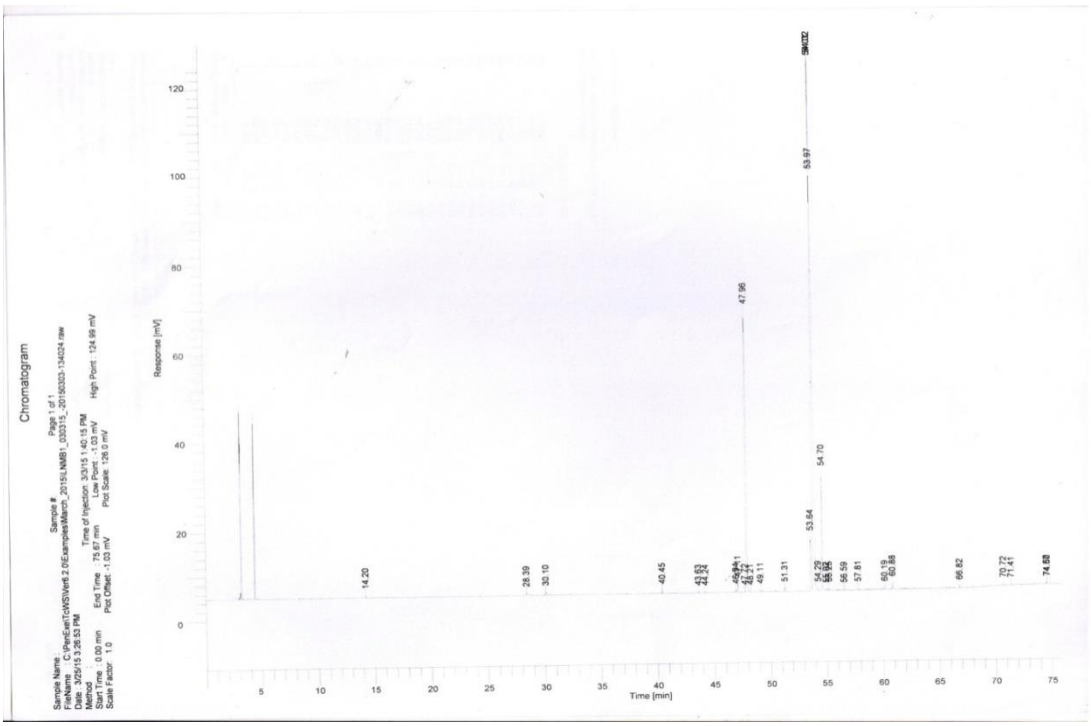
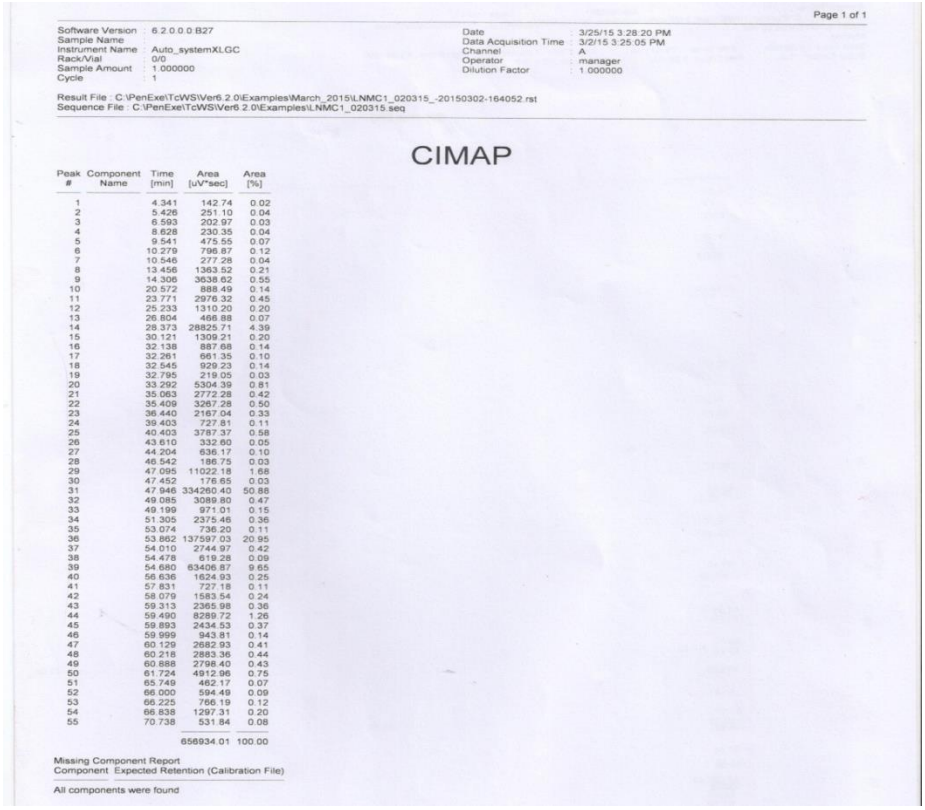


Fig. 23. GCMS of tasar silkworm pupal oil



Page 1 of 1

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 3:28:20 PM
Data Acquisition Time : 3/2/15 3:25:05 PM
Channel : A
Operator : manager
Dilution Factor : 1.000000

Result File : C:\PenExe\TcWS\Ver6.2.0\Examples\March_2015\LNMC1_020315_-20150302-164052.rst
Sequence File : C:\PenExe\TcWS\Ver6.2.0\Examples\LNMC1_020315.seq

CIMAP

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
1		4.341	142.74	0.02
2		5.426	251.10	0.04
3		6.593	202.97	0.03
4		8.628	230.35	0.04
5		9.541	475.55	0.07
6		10.279	796.87	0.12
7		10.546	277.28	0.04
8		13.456	1363.52	0.21
9		14.306	3638.62	0.55
10		20.572	888.49	0.14
11		23.771	2976.32	0.45
12		25.233	1310.20	0.20
13		26.804	466.88	0.07
14		28.373	28825.71	4.39
15		30.121	1309.21	0.20
16		32.138	887.88	0.14
17		32.261	661.35	0.10
18		32.545	929.23	0.14
19		32.795	219.05	0.03
20		33.292	5304.39	0.81
21		35.063	2772.28	0.42
22		35.409	3267.28	0.50
23		36.440	2167.04	0.33
24		39.403	727.81	0.11
25		40.403	3787.37	0.58
26		43.610	332.60	0.05
27		44.204	636.17	0.10
28		46.542	186.75	0.03
29		47.095	11022.18	1.68
30		47.452	176.65	0.03
31		47.946	334260.40	50.88
32		49.085	3089.80	0.47
33		49.199	971.01	0.15
34		51.305	2375.46	0.36
35		53.074	736.20	0.11
36		53.862	137597.03	20.95
37		54.010	2744.97	0.42
38		54.478	619.28	0.09
39		54.680	63406.87	9.65
40		56.636	1624.93	0.25
41		57.831	727.18	0.11
42		58.079	1583.54	0.24
43		59.313	2365.98	0.36
44		59.490	8289.72	1.26
45		59.893	2434.53	0.37
46		59.999	943.81	0.14
47		60.129	2682.93	0.41
48		60.218	2883.36	0.44
49		60.888	2798.40	0.43
50		61.724	4912.96	0.75
51		65.749	462.17	0.07
52		66.000	594.49	0.09
53		66.225	766.19	0.12
54		66.838	1297.31	0.20
55		70.738	531.84	0.08
			656934.01	100.00

Missing Component Report
Component Expected Retention (Calibration File)

All components were found

Fig.24. Retention time of eri silkworm pupal oil

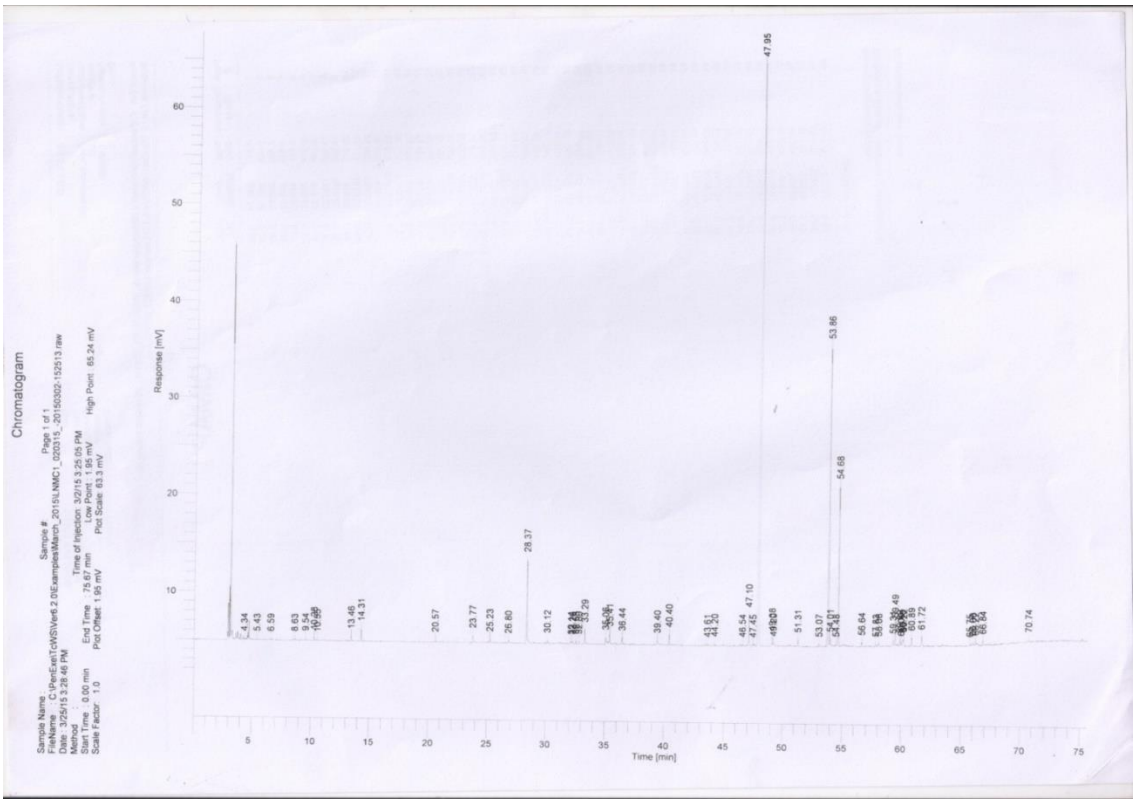
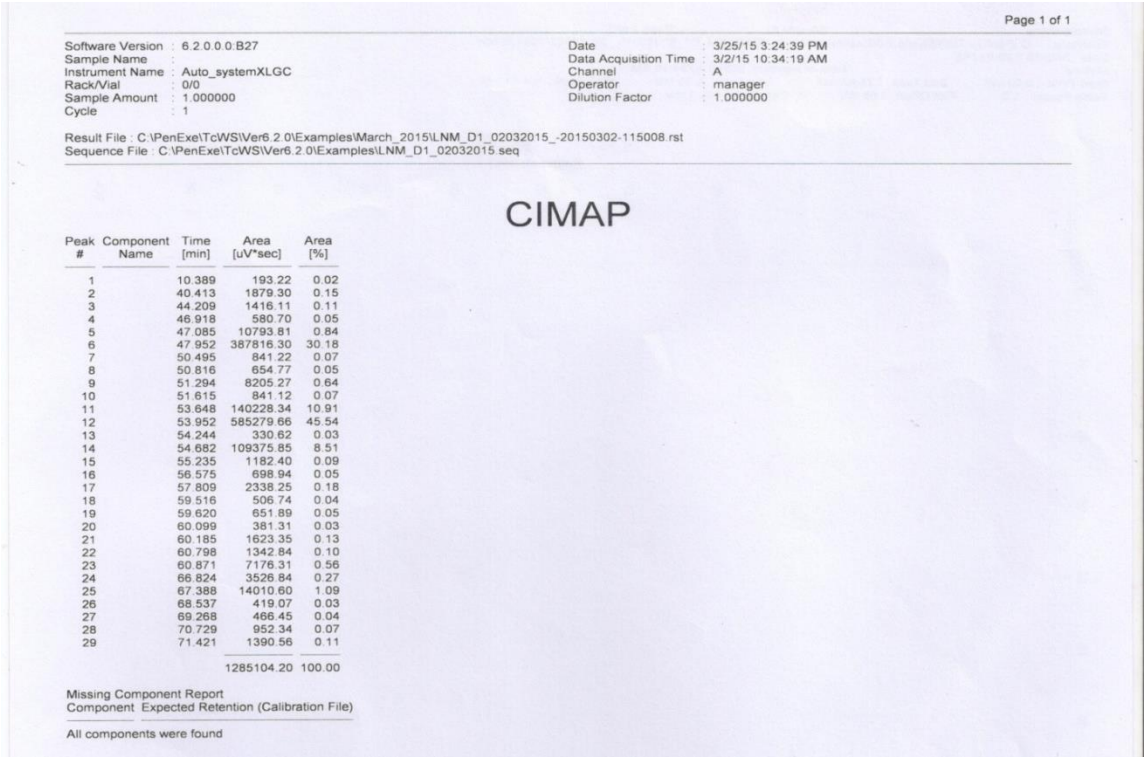


Fig.25. GCMS of Eri silkworm pupal oil



Page 1 of 1

Software Version : 6.2.0.0.0:B27
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Instrument Name : Auto_systemXLGC
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Cycle : 1

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Channel : A
Operator : manager
Dilution Factor : 1.000000

Result File : C:\PenExe\TcWS\Ver6.2.0\Examples\March_2015\LNM_D1_02032015_-20150302-115008.rst
Sequence File : C:\PenExe\TcWS\Ver6.2.0\Examples\LNM_D1_02032015.seq

CIMAP

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
1		10.389	193.22	0.02
2		40.413	1879.30	0.15
3		44.209	1416.11	0.11
4		46.918	580.70	0.05
5		47.085	10793.81	0.84
6		47.952	387816.30	30.18
7		50.495	841.22	0.07
8		50.816	654.77	0.05
9		51.294	8205.27	0.64
10		51.615	841.12	0.07
11		53.648	140228.34	10.91
12		53.952	585279.66	45.54
13		54.244	330.62	0.03
14		54.682	109375.85	8.51
15		55.235	1182.40	0.09
16		56.575	698.94	0.05
17		57.809	2338.25	0.18
18		59.516	506.74	0.04
19		59.620	651.89	0.05
20		60.099	381.31	0.03
21		60.185	1623.35	0.13
22		60.798	1342.84	0.10
23		60.871	7176.31	0.56
24		66.824	3526.84	0.27
25		67.388	14010.60	1.09
26		68.537	419.07	0.03
27		69.268	466.45	0.04
28		70.729	952.34	0.07
29		71.421	1390.56	0.11
			1285104.20	100.00

Missing Component Report
Component Expected Retention (Calibration File)

All components were found

Fig.26. Retention time of muga silkworm pupal oil

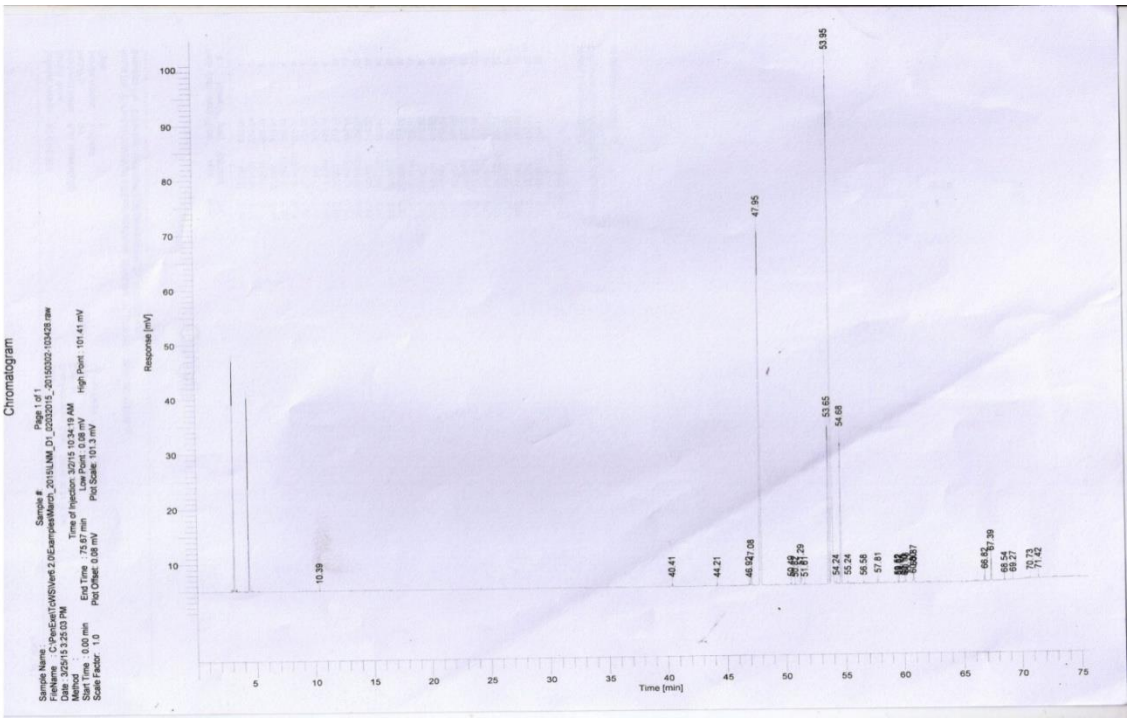

Fig.27. GCMS of muga silkworm pupal oil

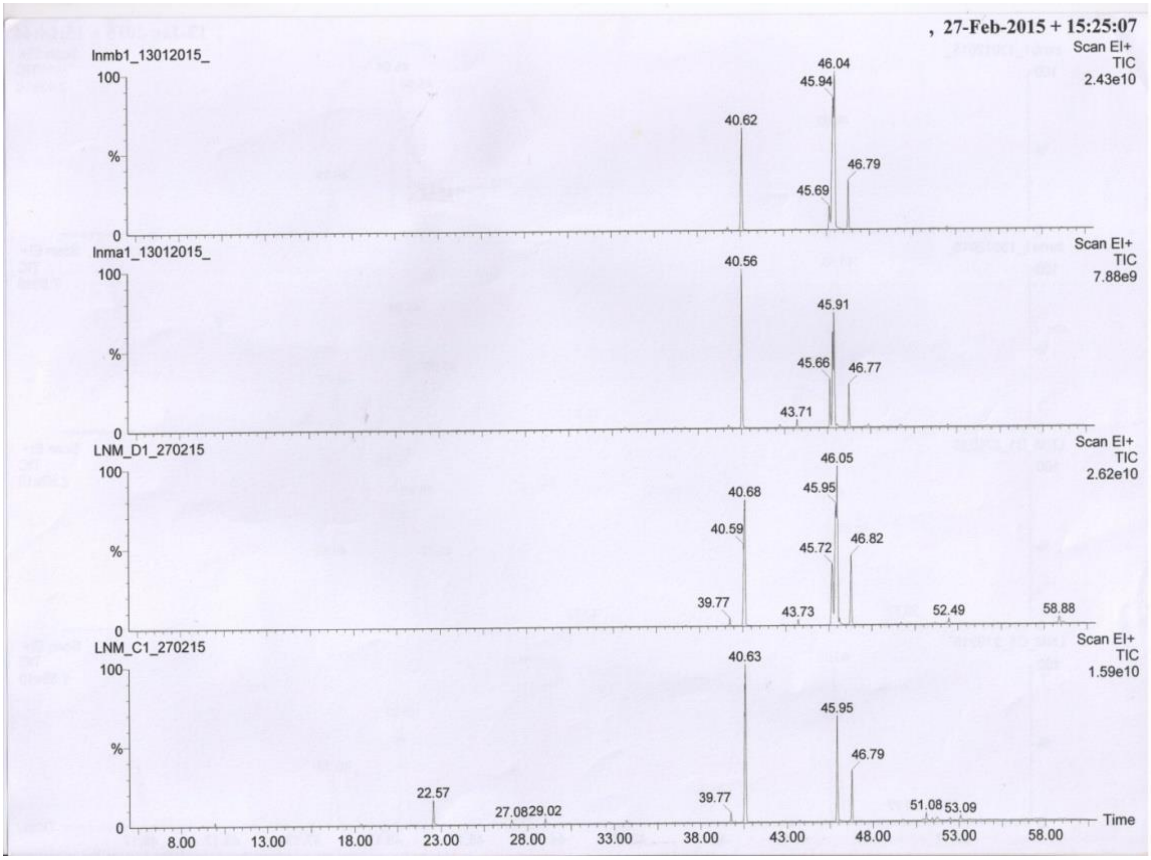

Fig. 28. GCMS analysis of mulberry and non mulberry silkworm pupal oil

Table 10. GC-MS analysis of mulberry silkworm pupae oil

Compound name	Retention time	Peak value	Area (uV*sec)	Area (%)
Palmitic acid	40.382	40.56	1000.46	0.65
Linoleic	44.200	45.66	698.91	0.46
Linolenic acid	47.090	45.91	1059.67	0.69
Stearic acid	49.083	46.77	392.46	0.26

Table. 11. GC-MS analysis of Tasar silkworm pupae oil

Compound name	Molecular Formulae	Molecular Weight g/mol	Retention time	Peak Value	Area (uV*sec)	Area (%)
Palmitic acid	$CH_3(CH_2)_{14}$ COOH	256.42	47.963	40.62	348174.25	23.54
Linoleic	$CH_3(CH_2)_4$ $CH=CHCH_2CH=$ $CH(CH_2)_7COOH$	280.44	53.637	45.69.	69816.92	4.72
Linolenic acid	$CH_3CH_2CH=$ $CHCH_2CH=$ $CH(CH_2)_7COOH$	278.43	53.970	45.94	474530.92	32.08
Oleic acid	$CH3(CH2)_7CH=CH(CH2)_7$ COOH	282.46	54.009	46.04	235074.89	15.89
Stearic acid	$CH_3(CH_2)_{16}$ COOH	284.47	54.022	46.79	212778.01	14.38

Table 12. GC-MS analysis of eri silkworm pupae oil

Compound name	Molecular Formulae	Molecular Weight g/mol	Retention time	Peak Value	Area (uV*sec)	Area (%)
Palmitic acid	$CH_3(CH_2)_{14}$ COOH	256.42	47.095	40.63	11022.18	1.68
Linolenic acid	$CH_3CH_2CH=$ $CHCH_2CH=$ $CH(CH_2)_7COOH$	278.43	53.862	45.95	137597.03	20.95

Stearic acid	$CH_3(CH_2)_{16}COOH$	284.47	54.680	46.79	63406.87	9.65

Table 13. GC-MS analysis of muga silkworm pupae oil

Compound name	Molecular Formula	Molecular weight	Retention time	Peak Value	Area (uV*sec)	Area (%)
Palmitic acid	$CH_3(CH_2)_{14}COOH$	256.42	47.952	40.68	387816.30	30.18
Linoleic	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	280.44	51.615	45.72	841.12	0.07
Linolenic acid	$CH_3CH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	278.43	53.648	45.95	140228.34	10.91
Oleic acid	$CH3(CH2)_7CH=CH(CH2)_7COOH$	282.46	53.952	46.05	585279.66	45.54
Stearic acid	$CH_3(CH_2)_{16}COOH$	284.47	54.244	46.82	330.62	0.03

Table 14. Determinations of physical and chemical characteristics of mulberry and non-mulberry silkworm pupae oils

Characteristic	Mulberry pupal oil mean±SD	Tasar pupal oil mean±SD	Eri pupal oil mean±SD	Muga pupal oil mean±SD
Density	0.742±0.0068	0.884±0.01	0.676±0.002	0.957±0.017
Viscosity	31.66±0.378	35.2±0.36	32.56±0.768	36.29±0.45
Acid Value	12.41±0.505	12.21±0.235	13.15±0.309	14.05±0.149
Iodine Value	130.55±0.579	134.05±0.201	136.21±0.1006	132.99±0.137
Ester Value	193.25±0.975	191.11±0.394	193.17±0.395	191.94±1.04
Saponification value	205.66±0.577	203.33±0.577	206.33±0.577	206±1.0
Unsaponifiable matter	0.164±0.0005	0.843±0.001	0.125±0.0015	0.883±0.1011

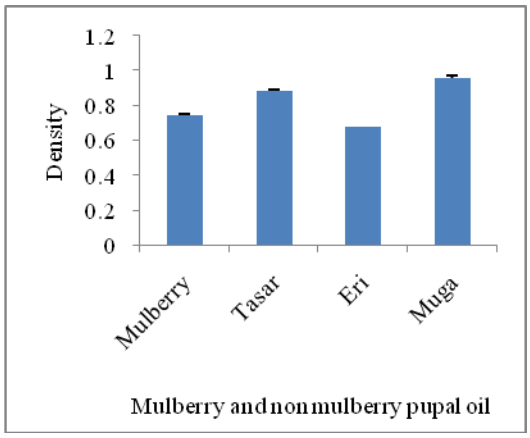

Fig 29. Density of mulberry and non mulberry silkworm pupae oils

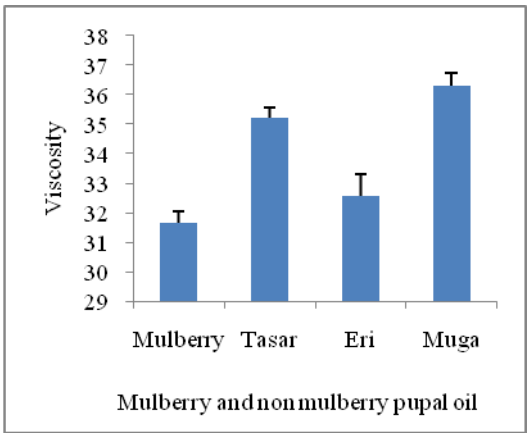

Fig 30. Viscosity of mulberry and non mulberry silkworm pupae oils

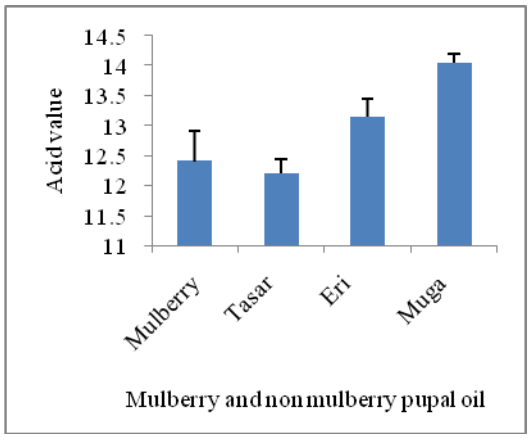

Fig 31. Acid Value of mulberry and non mulberry silkworm pupae oils

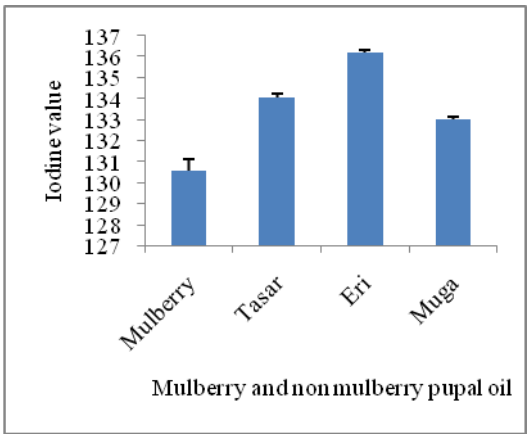

Fig 32. Iodine Value of mulberry and non mulberry silkworm pupae oils

3.5.2 Density and viscosity

Table 14 and Fig 29, 30 Showed the density and viscosity of mulberry, tasar, eri and muga silkworm pupal oil are 0.742±0.0068, 0.884±0.01, 0.676±0.002 and 0.957±0.017and 31.66±0.378, 35.2±0.36, 32.56±0.768 and 36.29±0.45 respectively.

3.5.3 Acid and iodine value

Table 14 and Fig 31, 32 indicate the acid and iodine value of mulberry, tasar, eri and muga are 12.41±0.505, 12.21±0.235, 13.15±0.309 and 14.05±0.149 and 130.55±0.579, 134.05±0.201, 136.21±0.1006 and 132.99±0.137 respectively.

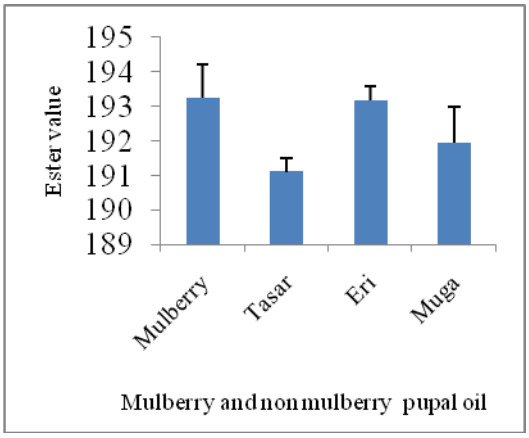

Fig 33. Ester value of mulberry and non mulberry silkworm pupae oils

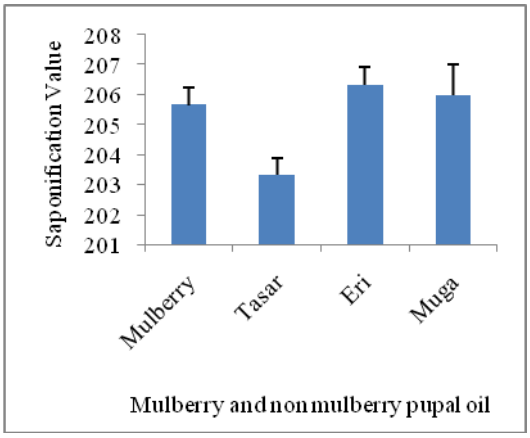

Fig 34. Saponification value of mulberry and non mulberry silkworm pupae oils

3.5.4 Ester and saponification value

Table 14 and Fig. 33, 34 showed ester and saponification value of mulberry, tasar, eri and muga silkworm pupal oil are 193.25±0.975, 191.11±0.394, 193.17±0.395 and 191.94±1.04 and 205.66±0.577, 203.33±0.577, 206.33±0.577and 206±1.0 respectively.

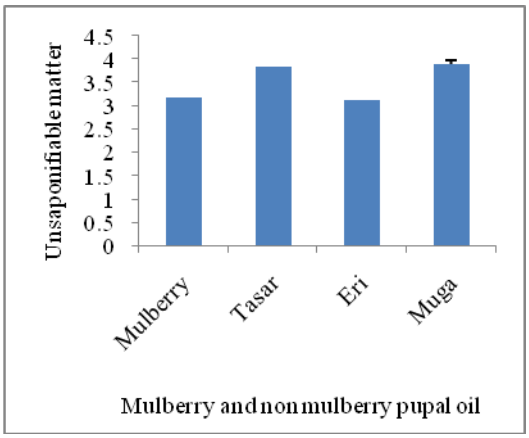

Fig 35. Unsaponifiable matter of mulberry and non mulberry silkworm pupae oils

3.5.5 Unsaponifiable matter

Table 14 and Fig. 35 showed unsaponification Matter of mulberry, tasar, eri and muga silkworm pupae oil are 0.164±0.0005, 0.843±0.001, 0.125±0.0015 and 0.883±0.1011 respectively.

After showing the results of physic-chemical properties of mulberry and non-mulberry silkworm pupae oils, further presentation of antioxidants activities of Mulberry, Tasar, Eri and Muga silkworm pupae oils.

Table 15. DPPH assay of mulberry and non-mulberry silkworm pupal oil

Treatment µl/ml	**Mulberry pupal oil**	**Tasar pupal oil**	**Eri pupal oil**	**Muga pupal oil**
Control	91.853±4.474	96.722±1.625	92.033±2.815	92.980±1.518
10µl	7.968±12.31	21.423±7.054	19.943±2.415	23.740±4.546
20 µl	28.058±17.64	38.426±11.300	38.95±11.465	42.656±11.774
30 µl	46.84±26.90	53.096±22.275	52.936±22.112	55.583±21.511
40 µl	66.149±4.477	68.683±5.996	71.166±4.315	73.246±4.596
50 µl	76.408±0.965	74.803±1.394	80.826±2.942	81.943±2.404
CV%	25.446	19.411	18.680	18.536
LSD $_{0.05}$	35.677	27.214	26.190	25.988
LSD $_{0.01}$	27.047	18.536	17.703	16.888

3.5.6 Antioxidant activity of mulberry and non mulberry silkworm pupae oils by DPPH assay method

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capacity of DPPH radical is determined by the decrease in its absorbance at 516 nm induced by antioxidants. The absorption maximum of a stable DPPH radical in ethanol was at 516 nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progresses, which result in the scavenging of the radical by the hydrogen donation. Table 15 and Fig.36, 37, 38, and 39 illustrates increase

scavenging of DPPH radical in dose dependent manner due to the scavenging ability of the mulberry and non-mulberry silkworm pupal oils.

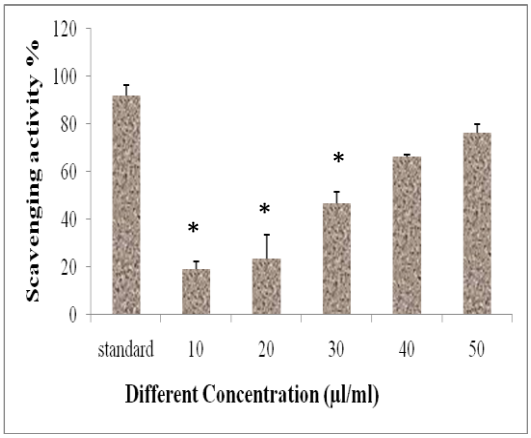

Fig 36. Antioxidant activities of mulberry silkworm pupal oil by DPPH assay method.

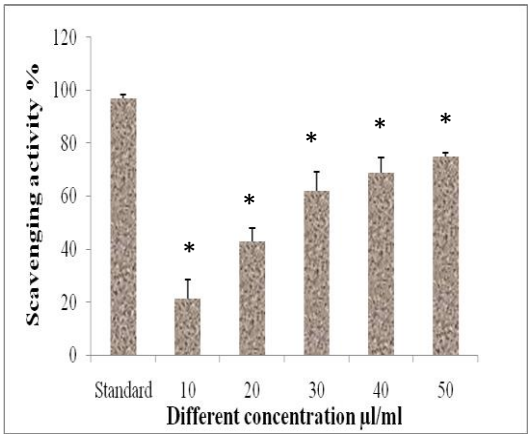

Fig 37. Antioxidant activities of tasar silkworm pupal oil by DPPH assay method.

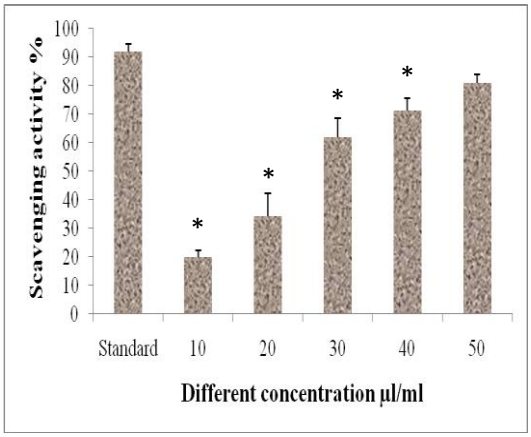

Fig 38. Antioxidant activity of eri silkworm pupal oil by DPPH assay method.

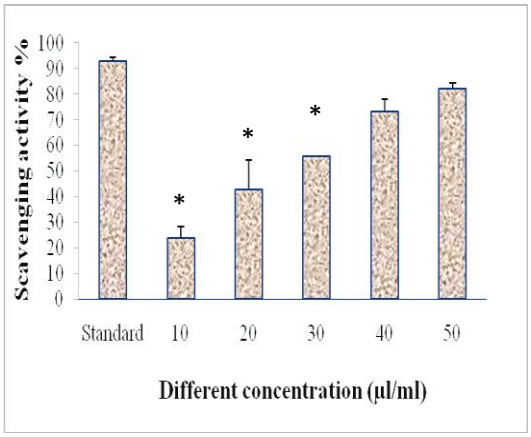

Fig 39. Antioxidant activity of muga silkworm pupal oil by DPPH assay method.

Table 16. FRAP assay of mulberry and non-mulberry silkworm pupal oil

Treatment µl/ml	Mulberry pupal oil	Tasar pupal oil	Eri pupal oil	Muga pupal oil
Control	0.251±0.009	2.062±0.045	2.094±0.070	2.123±0.015
10µl	0.047±0.007	0.822±0.154	0.265±0.013	0.210±0.130
20 µl	0.158±0.010	0.978±0.095	0.352±0.007	0.353±0.089

30 µl	0.116±0.007	1.225±0.126	0.377±0.006	0.385±0.086
40 µl	0.177±0.017	1.381±0.086	0.389±0.017	0.426±0.426
50 µl	0.197±0.017	1.446±0.054	0.440±0.030	0.478±0.043
CV%	0.021	0.179	0.058	0.143
LSD $_{0.05}$	0.030	0.252	0.082	0.201
LSD $_{0.01}$	7.787	7.662	5.036	12.160

3.5.7Antioxidant activity of mulberry and non mulberry silkworm pupae oils by FRAP assay method

In the present study, a concentration dependent ferric reducing antioxidant power (**FRAP**) of mulberry tasar, eri and muga silkworm pupal oils was observed with the highest activity of absorbance were 0.197±0.017, 1.446±0.054, 0.440±0.030 and 0.478±0.043µmol Fe (II)/g) at 50% concentration respectively shown in table 16 and fig. 40, 41, 42 and 43. β-carotene bleaching method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals which are formed by linoleic acid oxidation in an emulsion (**Kumazawa *et al.*, 2002)**. This fact is used in the antioxidant activity evaluation of essential oil and extracts of ginger. Antioxidant activity determined by β-carotene bleaching assay revealed that ginger essential oil had an antioxidant activity of 52.94% at 5% concentration and aqueous extracts had an antioxidant activity of 54.85% at 20% concentration. Results from the study agreement with similar results observed for oregano essential oil (**Kulisica *et al.*, 2004**). In comparison, the essential oil showed relatively significant ($p<0.05$) antioxidant effect. The antiradical efficiency of an effective antioxidant is measured in terms of its ability to counteract the higher levels of reactive radicals even at higher concentration (**Sanchez-Moreno *et al.*, 1998**).

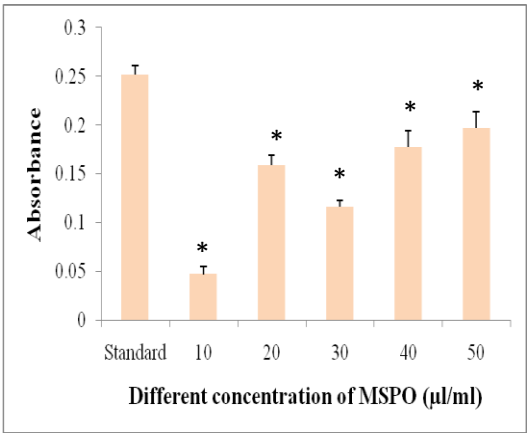

Fig 40. Antioxidant activity of mulberry silkworm pupal oil by FRAP assay method.

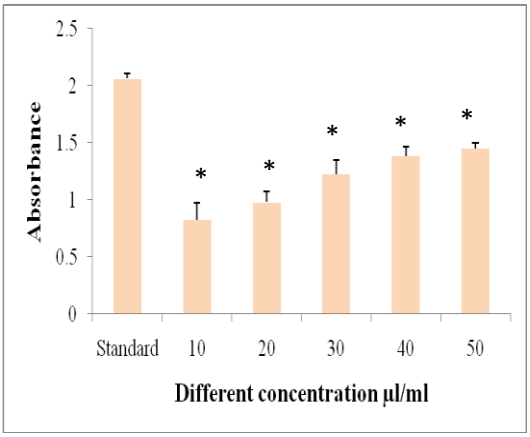

Fig 41. Antioxidant activity of tasar silkworm pupal oil by FRAP assay method.

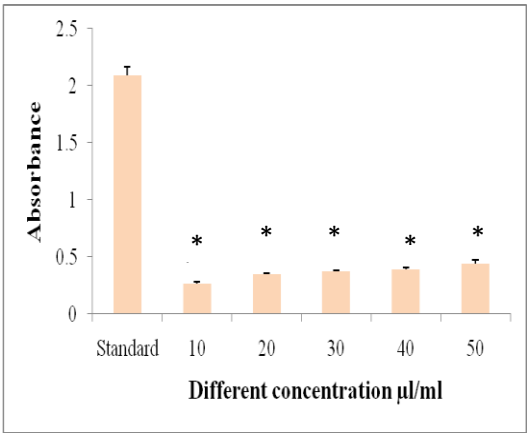

Fig 42. Antioxidant activity of eri silkworm pupal oil by FRAP assay method.

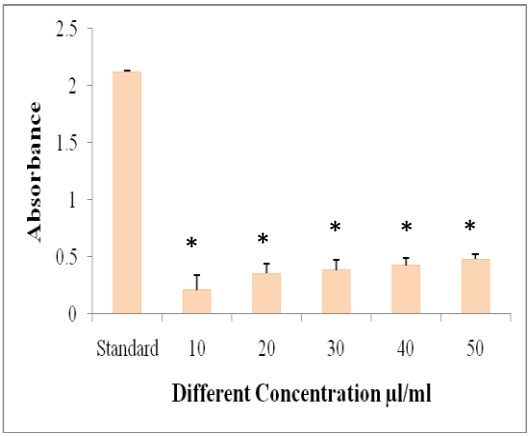

Fig 43. Antioxidant activity of muga silkworm pupal oil by FRAP assay method.

Table 17. Hydrogen peroxide assay of mulberry and non-mulberry silkworm pupal oil

Treatment µl/ml	Mulberry pupal oil	Tasar pupal oil	Eri pupal oil	Muga pupal oil
Control	90.021±3.144	82.333±2.46	81.48±2.303	80.211±2.971
10µl	15.858±5.835	23.422±2.376	13.389±6.45	11.781±1.8728

20 µl	24.685±8.575	34.725±2.935	22.103±2.489	21.58±1.876
30 µl	42.451±13.2	43.57±2.838	41.42±8.081	38.262±6.759
40 µl	54.046±6.574	57.33±1.151	55.156±3.787	52.273±7.202
50 µl	75.87±2.458	67.587±2.232	67.813±0.304	69.847±3.303
CV%	0.708	0.437	0.172	0.572
LSD $_{0.05}$	0.438	0.768	0.132	0.248
LSD $_{0.01}$	0.468	0.872	0.863	0.897

3.5.8 Antioxidant activity of mulberry and non mulberry silkworm pupae oils by hydrogen peroxide assay method

In this study, assessment of hydrogen peroxide assay of mulberry tasar, eri and muga silkworm pupal oils were significant ($p<0.05$) at 10µl/ml, 20µl/ml, 30µl/ml and 40µl/ml and 50 µl/ml concentration respectively showed in table 17 and figure 44, 45, 46 and 47. While the antioxidant enzymes such as SOD (Superoxide dismutase) and catalase constitute the major supportive team of defense against free radicals. SOD scavenges the superoxide radicals by forming H_2O_2 and molecular oxygen (**Karuna *et al.*, 2011**). Treatment with LUFO significantly normalized the SOD levels in dose dependent manner, suggesting the possible antioxidant potential of LUFO in counteracting STZ induced oxidative stress. Catalase is a hemeprotein, which catalyses the reduction of H_2O_2 (produced due to scavenging effect of SOD) and protects the tissue from highly reactive hydroxyl radicals. Thus simultaneous alteration in the catalase and SOD activity suggest the possible antioxidant potential of LUFO.

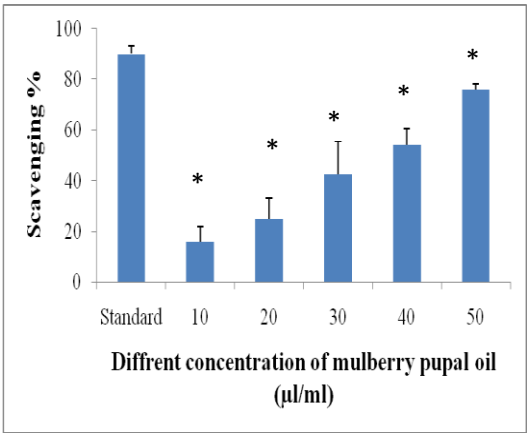

Fig44.Antioxidant activity of mulberry silkworm pupal oil by hydrogen peroxide assay method.

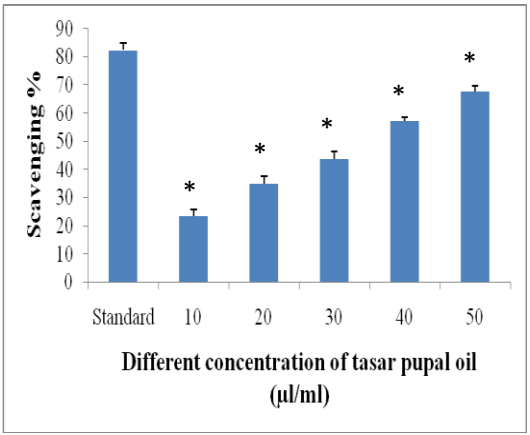

Fig 45. Antioxidant activity of tasar silkworm pupal oil by hydrogen peroxide assay method.

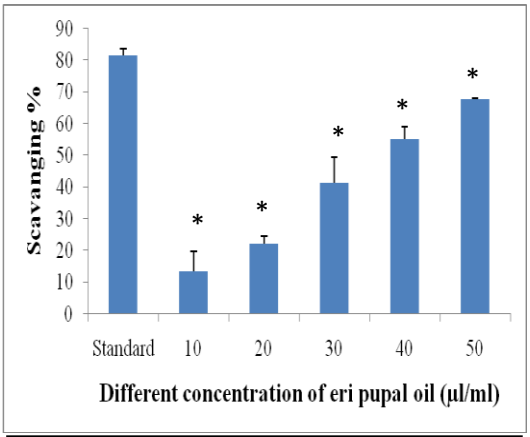

Fig 46. Antioxidant activity of eri silkworm pupal oil by hydrogen peroxide assay method.

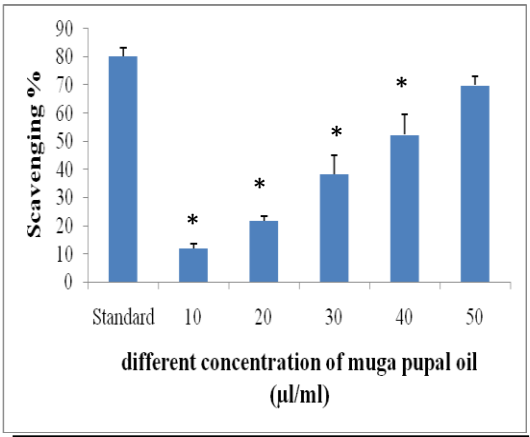

Fig 47. Antioxidant activity of muga silkworm pupal oil by hydrogen peroxide assay method.

Table 18. Superoxide Assay of mulberry and non-mulberry silkworm pupal oil

Treatment µl/ml	Mulberry pupal oil	Tasar pupal oil	Eri pupal oil	Muga pupal oil
Control	86.206±0.136	78.676±3.789	84.196±2.060	83.676±1.443
10µl	57.213±1.791	10.733±5.455	55.243±1.855	22.940±4.795
20 µl	57.203±1.645	8.896±0.351	63.943±0.943	71.723±0.213

30 µl	58.926±0.466	21.842±9.372	66.300±0.812	77.566±0.421
40 µl	77.583±0.080	40.366±1.665	71.506±0.540	79.040±0.365
50 µl	78.046±0.200	52.87±1.813	76.973±0.312	79.113±0.344
CV%	1.808	8.537	2.162	3.671
LSD $_{0.05}$	2.535	11.969	3.031	5.147
LSD $_{0.01}$	1.469	13.492	1.743	2.990

3.5.9 Antioxidant activity of mulberry and non mulberry silkworm pupae oils by superoxide scavenging assay method

Superoxide radicals are known to be very harmful to the cellular components. Superoxide free radicals were formed by alkaline DMSO which reacts with NBT to produce coloured diformazan. Mulberry and non-mulberry silkworm pupal oil scavenges superoxide radical and thus inhibits formazan formation. Table 18 and Figure 48, 49, 50 and 51 illustrates the increase scavenging of superoxide radical in dose dependent manner due to the scavenging activity of the mulberry and non-mulberry silkworm pupal oil.

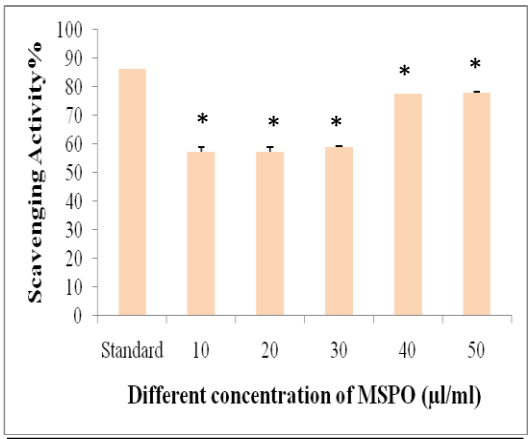

Fig 48. Antioxidant activity of mulberry silkworm pupal oil by superoxide assay method.

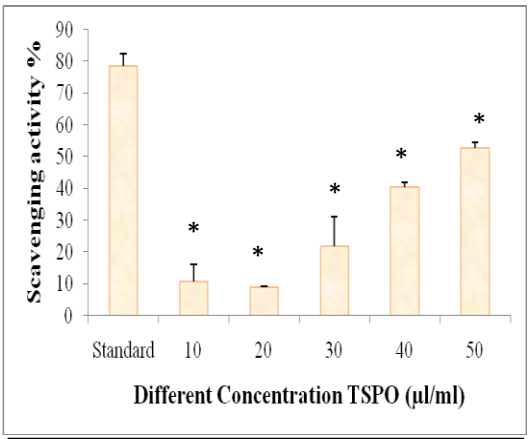

Fig 49. Antioxidant activity of tasar silkworm pupal oil by superoxide assay method.

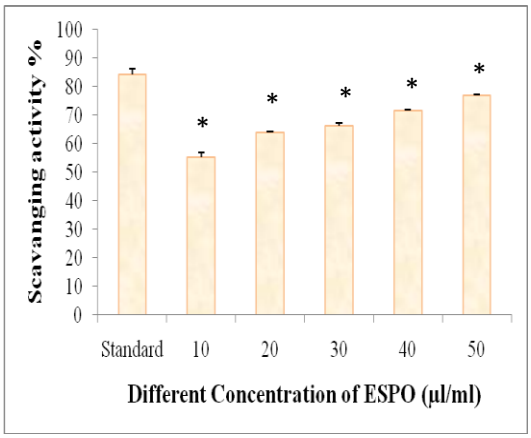

Fig 50. Antioxidant activity of eri silkworm pupal oil by superoxide assay method.

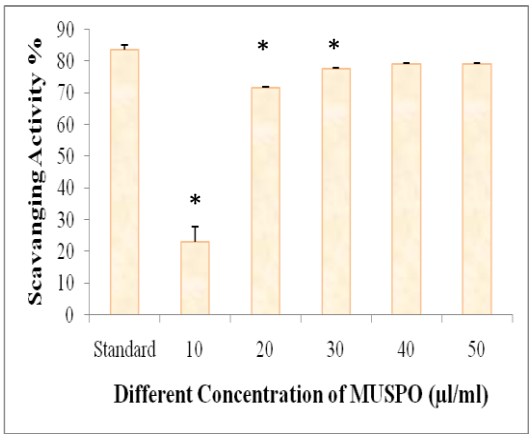

Fig 51. Antioxidant activity of muga silkworm pupal oil by superoxide assay method.

(3.679±0.179 at 50 µg/ml).

Table 19. Reducing Assay of mulberry and non-mulberry silkworm pupal oil

Treatment µl/ml	Mulberry pupal oil	Tasar pupal oil	Eri pupal oil	Muga pupal oil
Control	4.123±0.015	2.123±0.015	3.123±0.090	3.156±0.119
10µl	2.013±0.144	0.69±0.097	1.437±0.170	1.270±0.141
20 µl	3.083±0.213	0.836±0.078	1.745±0.411	1.645±0.320
30 µl	3.128±0.229	0.786±0.072	2.231±0.319	2.231±0.319
40 µl	3.699±0.006	0.885±0.061	2.713±0.418	2.713±0.418
50 µl	3.679±0.179	0.888±0.084	2.088±0.210	2.088±0.210
CV%	0.283	0.130	0.527	0.491
LSD $_{0.05}$	0.396	0.182	0.739	0.689
LSD $_{0.01}$	4.839	7.067	13.340	12.649

3.5.10 Antioxidant activity of mulberry and non mulberry silkworm pupae oils by superoxide reducing power assay method

In reducing power assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of the sample. The presence of reducing agents causes the conversion of Fe_3^+ferricyanide complex to the ferrous form that may be followed at 700 nm due to the formation of Perl's Prussian blue $Fe_4[Fe(CN)_6]_3$. Increasing absorbance at 700 nm indicates an increase in reducing ability (**Joshi *et al.*, 2010**). The antioxidants present in the mulberry, tasar, eri and muga silkworm pupal oils due to their reduction of Ferric (Fe_3^+) ferricyanide complex to the ferrous (Fe_2^+) form, and thus proved the reducing power. Table 19 and Figure 52, 53, 54 and 55 shows the reducing powers of mulberry and non-mulberry (tasar, eri and muga). It was found that the reducing power increased with concentration of the sample. The ranking orders for reducing power were for mulberry (50µl/ml), tasar (50µl/ml), eri (40µl/ml) and muga (40µl/ml) pupal oil. Significantly ($p<0.05$) higher reducing power of mulberry silkworm pupal oil was (3.679±0.179 at 50 µg/ml).

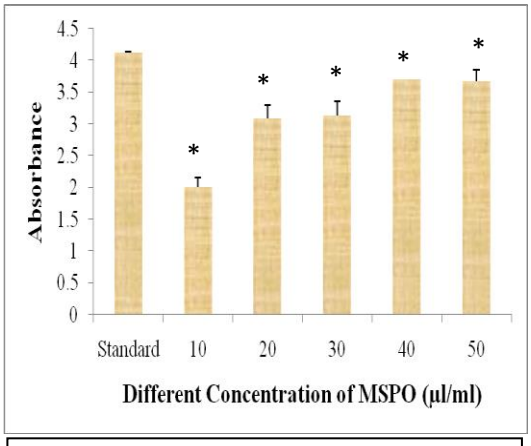

Fig 52. Antioxidant activity of mulberry silkworm pupal oil by reducing assay method.

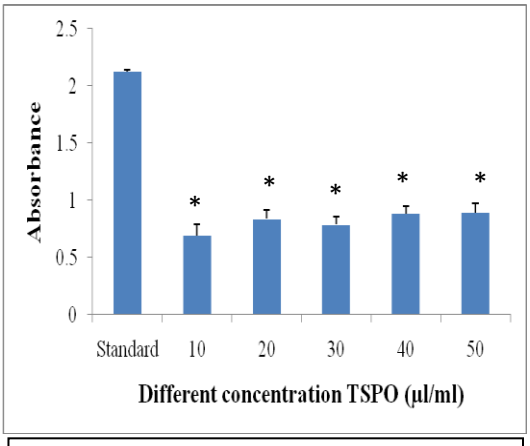

Fig 53. Antioxidant activity of Tasar silkworm pupal oil by reducing assay method.

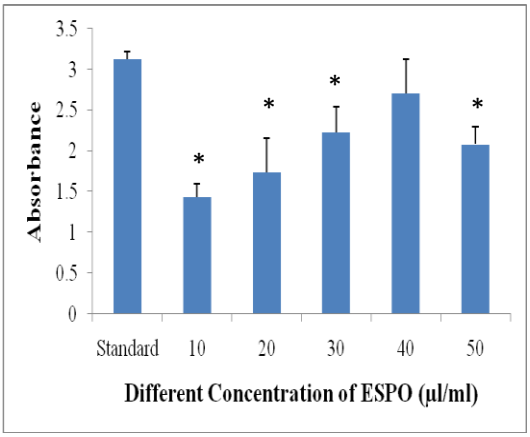

Fig 54. Antioxidant activity of Eri silkworm pupal oil by reducing assay method.

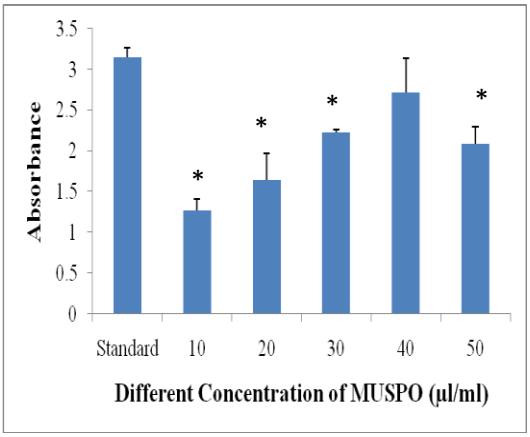

Fig 55. Antioxidant activity of Muga silkworm pupal oil by reducing assay method.

3.6 Discussion

Estimation of lipids is considered amongst the key factors for dietary assessment of every material (**Ayaz *et al.*, 2006**). However, the existence of a substantial content of lipids demonstrates the potential of any material to have dietary purposes with promising nutritional attributes.

Crude fat content of eri silkworm and muga silkworm pupae was around 20% and 16% respectively. The fat content of eri and muga silkworm pupae represents a good source of oil, which is comparable with the fat content of 20.1% in pupae of the silkworm *A. pernyi* (**Zhou and Han, 2006b**).

Oleic and linoleic acids as well as their derivatives were reported to exert a potent antioxidant effect in different assays (**Henry *et al.*, 2002, Shiota and Tatsumi 2002, Ceffarelli *et al.*, 2005, El-Din *et al.*, 2007, Hur *et al.*, 2007)**.

It was found that the oil yield decreases when the water content is either more or less than the optimum in the male and female pupae of eri silkworm. The physical and chemical properties such as color, odor, solubility, moisture content, refractive index, iodine value, saponification value, acid value and protein per cent were analyzed in male

and female pupae oil and have revealed not much remarkable variation. The color of the oil is light brown in male and female pupae during 0-216 hrs of development. It is found that the refractive index, iodine number and acid values vary among both sexes.

The refractive index of eri pupae oil is comparable to other common lipids of both vegetable and animal origin. This indicates the presence of long chain unsaturated fatty acids. The iodine value of eri pupae oil is quite high when compared to common animal lipids. The acid value indicates the presence of high amount of free fatty acids in eri pupae oil. Thus, eri pupae oil contains higher amount of unsaturated fatty acids than the common animal lipids.

The saponification value signifies that its ransidification values are lower than that of common animal fats. The analysis of the results such as that of eri pupae oil has great prospect for its utility in food industry as well as the source material for oleo-chemical industries. This observation coincides with that of **Choudhury (2003)**.

The eri pupae oil is safe, nutritionally equivalent and commonly used as vegetable oil. Eri silkworm pupae can be harvested to provide cost effective alternate edible oil that can be used as nutritional advantages in the food and feed industry. Therefore, eri silkworm and its host plants offer an excellent example of multiple producing crops and of sustainable agricultural practice with excellent opportunity for economical and nutritional benefits **(Thingnganing Longvah *et al.*, 2012)**.

Physicochemical Properties Of Eri Pupae Oil studied by Sharma & Ganguly and found the viscosity 33.6%, Iodine value 128%, Saponification number 218%.**Thumu Ravinder *et al* (2016)** also studied physico-chemical properties of eri silkworm pupal oil and found the density, viscosity, iodine value, saponifiable value and unsaponifiable

matter were 0.9126±0.10, 32.21±0.14, 130.90±0.31, 196.45±0.28, 3.33±0.41 respectively. While physico-chemical properties in case of mulberry (density 0.742±0.0068, viscosity 32.5±1.053, iodine value 132.08±1.799, saponifiable value 205.66±1.527 unsaponifiable matter 0.164±0.0005), tasar (density 0.884±0.01, viscosity 35.86±1.28, iodine value 133.85±2.504, saponifiable value 205±2.00unsaponifiable matter 0.843±0.001) eri (density 0.676±0.002, viscosity 33.56±2.35, iodine value 133.88±2.019, saponifiable value 205±2.645unsaponifiable matter 0.125±0.0015) and muga (density 0.957±0.017, viscosity 34.62±0.673, iodine value 133.56±1.659, saponifiable value 206.66±1.527unsaponifiable matter 0.883±0.1011) silkworm pupal oil respectively.

Meetali et al (2014) investigate the antioxidant activity of pupae of the eri (*Samia ricini*) and muga silkworm's (*Antheraea assamensis)* methanolic pupae extract (MPE). Antioxidant activity was determined by using 1-1 diphenyl 2 picryl hydrazyl (DPPH) radical, and reducing power assay method. The methanolic pupae extract (MPE) of eri and showed good DPPH radical scavenging activity with IC_{50} value of 18.71µg/mL and 25.83µg/mL respectively. The MPE of muga pupae had phenolic (12.2 mg catechin/g) and flavanoid content (5.45 mg quercetin/g) ($p < 0.05$) and MPE of eri pupae had significant higher phenolic (17.69 mg catechin/g) and flavanoid content (3.47 mg quercetin/g) ($p < 0.05$). Therefore, pupae could be used as natural antioxidants on food products.

Abdalbasit Adam Mariod studied on *Aspongopus vidiuatus* (melon bug) and *Agonoscelis pubescens* (sorg-hum bug) insects and found that theses two insect showed 27.0% and 28.2% crude protein, 45% and 60% oil, respectively. The oils contained

46.5% and 40.9% oleic acid, 3.4% and 34.5% linoleic acid, 44.2% and 12.1% palmitic acid and traces of linolenic acid, respectively. The tocopherol content of these oils amounted to 0.3 and 34.0 mg/100g oil, respectively. The total content of sterols in the two oils was 17 and 450 mg/100g oil, respectively, whereas β-sitosterol was determined as the main compound in all oils with about 60% of the total sterol. The oxidative stability of the oils, as measured by the Rancimat test at 120˚C, was 38 and 5.1 h, respectively.

Aspongubus viduatus and A. pubescens have 37.9% and 20.5% of saturated fatty acids (SFA), 56.8% and 43.0% of monounsaturated fatty acids and 5.3% and 36.5% of polyunsaturated fatty acids (PUFA), respectively. The balanced ingestion of foods containing PU-FAs reduces cardiovascular disorders. The PUFA: SFA ratio in the oil of A. viduatus is relatively lower (about 0.14), while it is relatively high (about 1.78) in the case of A. pubescens. This ratio is recommended to be 0.45 for a healthy diet; so A. viduatus oil seems to be healthier than that of A. pubescens. In addition, the two insect oils have a n-6/n-3 fatty acids ratio (10.8 and 27.5 for A. vi-duatus and A. pubescens, respectively) that should be smaller than 4.0 as suggested by the UK Department of Health (**Mariod *et al.*, 2011**). The low amounts of polyunsaturated fatty acids such as linoleic and linolenic acid in insect oils give them high oxidative stability. The fatty acid composition has a much higher influence on the stability of these oils than the minor components of antioxidants present in the oil.

Kaithwas (2012) evaluate the antioxidant, effect of *Linum usitatissimum* fixed oil. The antioxidant activity was evaluated in vitro using 1, 1-diphenyl-2-picryl hydrazyl

(DPPH) and hydrogen peroxide (H_2O_2) assay. The oil exhibited significant in vitro antioxidant activity in the DPPH and H_2O_2 assay.

DPPH, a stable free radical with characteristics absorption at 517 nm in methanol was used to study the radical scavenging effects of MPE (methanolic pupae extract) of the muga and eri silkworm. As antioxidants donate proton to this radical, the absorption of scavenging activity of DPPH decreases. The decrease in the absorbance at 517 nm is taken as a measure of the extent of radical scavenging. The IC_{50} values (the concentration with scavenging activity of 50%) of scavenging activities on DPPH radical of MPE of muga and eri silkworm were found to be 25.83µg/mL and 18.71µg/mL respectively.

The change of reducing power with different concentrations of MPE of the muga and eri silkworm is evaluated and is presented in Table 3. Trolox was used as control, and reducing power of MPE of muga at 80µg/mL is 0.353. Higher absorbance indicates higher reducing power.

Meetali Deori (2014) studied on antioxidant activities of muga and eri silkworm pupae by organic radical DPPH· has been widely used in the determination of antioxidant activity of extract following the method of Brand William method (**Brand- William *et al.*, 1995**). The solution of DPPH in methanol (6×10-5 M) was prepared just before UV measurements. Samples were added to DPPH solution in 1:1 ratio followed by vortex. The reaction was allowed to take place in the dark at room temperature under nitrogen atmosphere. The absorbance at 517 nm was measured at different time intervals. A decreasing intensity of the purple colour was taken as increasing scavenging activity. Ascorbic acid served as a standard. The inhibition percentage of radical scavenging activity was calculated using the following equation.

Inhibition (%) = [(A0-A)/A0] ×100

Where A0 is the absorbance of DPPH· in the absence of the sample and A is the absorbance of DPPH· in the presence of sample.

DPPH a stable free radical with characteristics absorption at 517 nm in methanol was used to study the radical scavenging effects of MPE of the muga and eri silkworm. As antioxidants donate proton to this radical, the absorption of scavenging activity of DPPH· decreases. The decrease in the absorbance at 517 nm is taken as a measure of the extent of radical scavenging. The IC50 values (the concentration with scavenging activity of 50%) of scavenging activities on DPPH· radical of MPE of muga and eri silkworm were found to be 25.83 μg/mL and 18.71μg/mL respectively.

Meetali Deori (2014) studied on antioxidant activities of muga and eri silkworm pupae by reducing power was determined according to the **Oyaizu (1988**). 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1 % potassium ferricyanide were added to 1 mL sample solution and mixed gently. The mixtures were incubated at 50˚C in a water bath for 20 min. Reaction was stopped by adding 2.5 mL of 10 % trichloroacetic acid (TCA) and the mixtures were centrifuged at 4000 rpm for 10 min. From the top layer, 2.5 mL was transferred into tubes containing 2.5 mL distilled water and 0.5 mL of 0.1% ferric chloride ($FeCl_3.6H_2O$). The resulting solutions were mixed well and after 5 min the absorbance was measured at 700 nm against blanks.

The change of reducing power with different concentrations of MPE of the muga and eri silkworm is evaluated and is presented in Table 3. Trolox was used as control, and reducing power of MPE of muga at 80μg/mL is 0.353. Higher absorbance indicates higher reducing power.

However, the activity of antioxidant has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxide, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging activities (**Diplock, 1997**). Reducing power is widely used to evaluate the antioxidant activity of polyphenols. The reducing power of a compound is related to its electron transfer ability and may, therefore serves as a significant indicator of its antioxidant activity (**Ajila *et al.*, 2007**). The phenolic compounds were directly correlated with its antioxidant ability. The physiological effects of flavanoid include possible antioxidant activity, therefore, suggesting their role in prevention of coronary heart diseases including atherosclerosis (**Sierens *et al.*, 2002**). Lipids, especially polyunsaturated fatty acids, are sensitive to oxidation, leading to the formation of malondialdehyde (MDA). **Winitchai *et al.* (2011**) extracted oil from five native Thai silkworm varieties. The oil extracted by the Soxhlet method from silkworm varieties showed free radical scavenging activity. **Meetali *et al.* (2014)** investigated antioxidant activity of pupae of the muga and eri silkworm and concluded that, the pupae could be used as natural antioxidants on food products.

3.7 Conclusion:

Analysis of mulberry and non mulberry pupal oil properties showed fatty acids, such as alpha linolenic acid, in mulberry, tasar, eri and muga were 0.69%, 32.08%, 20.95% and 10.91% respectively. This fatty acid is an essential fatty acid that cannot be naturally synthesized in the body and so has to be acquired from nutrition.

The results presented here can provide evidence that the mulberry and non mulberry silkworm pupae oils could be used in food industries and other fields, which

process natural products. However, further studies are certainly needed for a better clarification of the potential and its biological effects of the silkworm pupae oils presented here.

CHAPTER: IV

ANTIDIABETIC AND ANTIHYPERLIPIDEMIC ACTIVITY OF MULBERRY AND NON MULBERRY SILKWORM PUPAE OILS

4. Introduction

4.1 Background of the study

Diabetes mellitus (DM) is considered as a widespread, budding, severe, expensive and potentially unnecessary public health problem. In other words, DM is a common chronic metabolic disease, usually caused by the interaction of genetic and environmental factors (**American Diabetes Association., 2012**). It is characterized by a lack of insulin secretion and insulin resistance (**International Diabetes Federation., 2011**), always leading to metabolism disorders of fat, protein and carbohydrate (**Szybinski., 2001**), and is likely to produce serious complications concerning some of the vital organs, together with the heart, blood vessels, nerves, eyes and kidneys as well as causing tissue lesions (**National Institutes of Health., 2012**). It is estimated that people with diabetes are predicted to increase from 117 million in 2000 to 366 million till 2030 (**Adams *et al.*, 2011**). There are approximately 366 million people worldwide who suffer from diabetes and another 280 million people with pre-diabetes as evidenced by impaired glucose tolerance. In 2011, 4.6 million people died from diabetes, meaning one diabetes-related death every seven seconds (**International Diabetes Federation., *et al.*, 2011**). The pervasiveness rate is 8.9%-12.3% in human population (**Zangiabad *et al.*, 2007**). Diabetes is a general metabolic disorder of all three of the energy nutrients fat, carbohydrate and protein but it is mainly due to carbohydrate metabolism (**Susan., 2006**). In 2008, the prevalence of diabetes in Egypt was 4.07%. In 2010, the international incidence of diabetics among adults has been estimated to be 6.4%. In 2030, Egypt will have at least 8.6 million adults with diabetes (**Arafa and Amin., 2010**). Diabetes is two type one is type-1 diabetes and another is type-2 diabetes, wherein more than 90% of all people with diabetes have type-2 diabetes. The treatment of type-1 diabetes is mainly dependent on exogenous insulin (**Hutton and Davidson., 2010**), whereas the treatment

of type 2 diabetes often include biguanides, sulfonylureas, α-glucosidase inhibitors, and other drugs (**Lundgren *et al.*, 2010**).

4.2 Antidiabetic property of DNJ

The diabetes mellitus (DM) is also well known to damage vascular function and is linked with increased risk of cardiovascular diseases (CVD) **(Blaak *et al.*, 2012).** Current scientific facts show a difficult association between hyperglycemia and forceful treatment with natural products possessing potent α-glycosidase inhibitory activity **(Huang *et al.*, 2013, Hou *et al.*, 2009, Yatsunami *et al.*, 2008).** Furthermore, the possibility of preventing the onset of diabetes using dietary supplements and/or herbal medicines has concerned substantial consideration **(Kimura *et al.*, 2007).** This suggests that the bioavailability of DNJ might not be enough through an oral ingestion of mulberry leaves. Consequently, other natural resources whose DNJ levels are adequate for regulating blood glucose are being explored. The DNJ found in silkworms comes from the mulberry leaf**.** Numerous studies in Korea have shown that silkworm powder and extract exhibited a significant hypoglycemic consequence both in mice fed with a high carbohydrate-containing diet **(Chung *et al.*, 1997)** and in alloxan-induced diabetic mice **(Ryu *et al.*, 1997).** Another interesting result showed that DNJ can be concerted numerous folds by silkworms feeding on mulberry leaves **(Asano *et al.*, 2001, Yin *et al.*, 2010, Liu *et al.*, 2013).**

After feeding mulberry and non-mulberry leaves by the larvae of mulberry, tasar, eri and muga converted in to pupae. In the pupae oils it was found that ALA was presented which ALA (18:3 n-3) is metabolized to EPA (20:5 n-3) and DHA (22:6 n-3) by series of desaturation and

elongation reactions. EPA (20:5 n-3) and DHA (22:6 n-3) are essential PUFA (Polyunsaturated fatty acids) and are the important component of cell membranes.

4.3 Role of PUFA to control diabetes

The diabetes is increasing among the people due to ingestion of saturated fatty acids. Now the researcher has found that diabetes can be control by using unsaturated fatty acids in the meal. So, there are three types of omega-3 in foods. The short chain is alpha linolenic acid (ALA): 18:3n-3 (Cis-9,12,5, octadecatrienoic acid), and long chain is eicosapentaenoic acid (EPA): 20:5n-3 (Cis-5,8,11,14,17-eicosapen- taenoic acid) and docosahexaenoic acid (DHA): 22-6n-3 (Cis-4,7,10,13,16,19 docosahexaenoic acid).The major sources of ALA are vegetable oils (such as canola and soybean) with other sources being flaxseed oil. Flaxseed is alternative to plant source of omega-3 fatty acid, which is one of the richest plant sources of omega-3 fatty acid alpha-linolenic (ALA, C18:3n-3) (**Rodriguez-Leyva *et al.*, 2010**).

4.4 Antidiabetic Property of omega-3 fatty acids

The bioavailability of ALA is greater in oil than in milled seed (**Austria *et al.*, 2008**). ALA is considered to be nutritionally essential due to its explicit function as the precursor of the long-chain n-3 (omega-3) PUFA (**Bresson *et al.*, 2000**). The consumption of ALA was related to higher plasma insulin and improves glucose used and efficiency (**Djousse *et al.*, 2001**). EPA and DHA act as hypolipidamics which protect against insulin resistance and obesity in rodent fed high diet and insulin response to glucose in healthy human being (**Ruzickowa *et al.*, 2004, Delarue *et al.*, 2000**). Polyunsaturated fatty acid (PUFA) as EPA and DHA which play a key role in normal

development and functioning of the brain and central nervous system (**Schuchardt *et al.*, 2010**).

Studies have also suggested that fatty acid composition of skeletal muscle phospholipids has been related to peripheral insulin sensitivity, thus BGL (**Cortright *et al.*, 1997**). Increased PUFA [i.e., EPA (20:5 n-3) and DHA (22:6 n-3)], provide higher membrane fluidity, increase in number of insulin receptors and insulin action and thus improve glucose homeostasis **(Gorjao *et al.*, 2009).** The present study is Antidiabetic and anti-hyperlipidemic activity of mulberry and non-mulberry silkworm pupal oil.

4.5 Materials and Methods

4.5.1 Drugs and chemicals

All chemicals used were of analytical grade. Streptozotocin (STZ) was obtained from Bionic Enterprises, B-28, Madhav Complex, Ram Nagar, Alambagh, Lucknow-226005.

4.5.2 Experimental design

The 20 female albino wistar rats weighing between 25-50 grams were obtained from CSIR-Central Drug research institute, New Campus Jankipuram Extension Lucknow; 34/99/CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). After necessary approval of Institutional Animal Ethical Committee all experimental procedures and laboratory animal handling were carried out carefully in strict accordance. Throught the study rats were allowed food and water ad libitum.

The animal housed individually in stainless steel cages under controlled condition at constant temperature (22-25°C) and lighting 12 h. light-dark cycle and given free

access to food and water at all time. The rats were divided randomly into five groups; each group fed the following diets for 8 weeks:

4.5.3 Induction of diabetes

A group of rats were rendered diabetic by single intravenous injection of 50 mg/kg of STZ (Streptozotocin) dissolved in 0.1 M citrate buffer (pH 4.5). Diabetes was confirmed in STZ treated rats by measuring the fasting blood glucose concentration 96 h after the injection of STZ. The rats with blood glucose level (BGL) >200 mg/dL (milligrams per deciliter) were considered to be diabetic and were used in the experiment

Group normal 1: Rats were fed on standard diet mentioned as normal control (**negative control group**). Standard diet was prepared according to (**Gaithersburg, 2000**). It contained 14% casein, 2% cellulose, 5% salt mixture, 1% vitamin mixture, 0.25% choline, 0.3% Dl-methionine, 8% corn oil and remaining percentage was starch.

Group 2: Rats were fed on fructose rich diet + 8% mulberry silkworm pupal oil

Group 3: Rats were fed on fructose rich diet + 16% mulberry silkworm pupal oil

Group 4: Rats were fed on fructose rich diet + 24% mulberry silkworm pupal oil

Group 5: Rats were fed on fructose rich diet + 32% mulberry silkworm pupal oil

Each rat was weight at the initial and the end of experimental and food intake also was recorded daily. Blood glucose level was estimated using one-touch glucometer on 0, 7, 14, and 21st day. Blood glucose was measured using one touch glucometer.

4.5.4 In vitro antihyperlipidemic assessment of mulberry and non mulberry silkworm pupae oils

Further for in-vitro assessment the albino rats weighing between 180-250 grams were kept in animal housed individually in stainless steel cages under controlled

condition at constant temperature (22°C) and lighting 12 h. light-dark cycle) and given free access to food and water at all time.

Pic 12. Anti-hyperlipidemic activity of the pupal oil by using organ bath

Further for in-vitro analysis of rats Tyrode's solution were prepared by using following chemical as: Nacl 8.0gm, Kcl 0.2 gm, $Cacl_2$ 0.2gm, $NaHCo_3$ 1.0gm, NaH_2Po_4 0.05gm, Glucose 1.0gm by dissolving in one liter double distilled water solution.

In-vitro analysis the sample (mulberry and non mulberry silkworm pupal oil) were prepared by using surfactant. Weighing 180-250g rats were sacrifice and their ileums were cut out and placed in oxygenated Tyrode's solution at room temperature. The ileum was then cut to 4-5 cm long four strips and mounted in 50 ml organ bath filled with Tyrode's solution at 37°C and continuously bubbled with O_2 and tyroid solution were kept on magnetic stirrer. Further mulberry and non-mulberry silkworm pupal oil (1-1 ml) were filled into the four different piece of intestine. Further, these intestines were tightly bind with thread from both the sides and kept into beaker filled with Tyrode's solution. The samples were taken out at different-different time and results are presented in Table 21 (**Kaithwas and Majumdar 2012**).

4.6 **Results:**

The blood glucose levels of control groups of rats were as 144.333±1.527, 147.666±1.527, 151.000±1.000 and 156.666±1.5275. After induction of STZ (Streptozotocin) diabetic the blood sugar level increases as 343.333±18.823, 282.666±45.346, 241.000±12.165 and 211.000±22.538. The different concentration as 8mg/ml, 16mg/ml, 24mg/ml and 32mg/ml mulberry and non-mulberry (tasar, eri muga) silkworm pupal oil given orally to diabetic rats. The result demonstrated that there are significant different ($P \leq 0.05$) between the blood glucose levels between control and treated groups in table 20 and figure 56, 57, 58 and 59.

Table 20. Experimental analysis of diabetic mice treated by mulberry and non-mulberry (Tasar, Eri, Muga) silkworm pupal oil

Treatment mg/ml	**Mulberry pupal oil**	**Tasar pupal oil**	**Eri pupal oil**	**Muga pupal oil**
Control	144.333±1.527	147.666±1.527	151.000±1.000	156.666±1.5275
8mg	343.333±18.823	282.666±45.346	241.000±12.165	211.000±22.538
16mg	173.666±14.843	188.000±2.000	207.000±16.462	185.666±11.590
24mg	177.000±10.440	165.666±21.126	190.666±7.371	157.666±28.005
32mg	176.333±9.451	177.333±19.655	193.666±3.511	170.333±25.696
CV%	22.154	42.813	17.552	36.368
LSD $_{0.05}$	31.061	60.024	24.609	50.989
LSD $_{0.01}$	6.136	12.515	5.016	11.597

The increasing concentration of mulberry, tasar, eri and muga silkworm pupal oil lowering the blood glucose level as on 16mg/ml the blood sugar level were as 173.666±14.843, 188.000±2.000, 207.000±16.462 and 185.666±11.590 respectively.

While at 32mg/ml the blood sugar level increases for tasar, eri and muga except mulberry silkworm pupal oil. Silkworm pupal oil have omega-3 fatty acids like ALA, EPA and DHA. Fish oil also has high concentration of long chain omega-3 fatty acid 39.20% (23.98% of EPA and 15.22% of DHA). These results are concord with **(James and Cleland., 2000, 42, Makrides *et al.*, 1995)** who mentioned that the long chain polyunsaturated fatty acid was found mainly in fish and fish oil. Fish and fish oil are the only major dietary source for human of omega-3 long chain fatty acid **(Campioli *et al.*, 2012).**

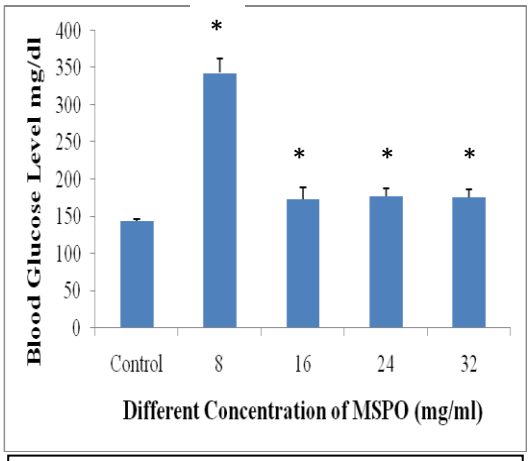

Fig 56 Diabetic mice treated by mulberry silkworm pupal oil and blood glucose level in mg/dl

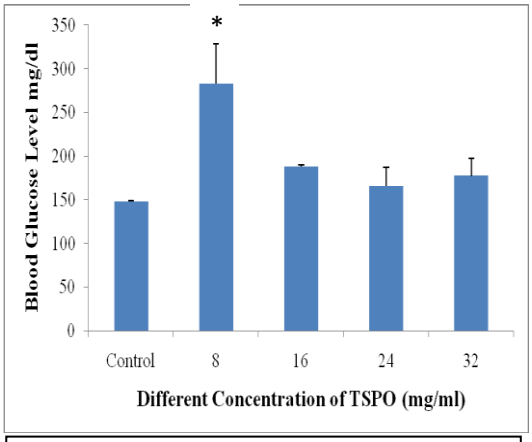

Fig 57. Diabetic mice treated by tasar silkworm pupal oil and blood glucose level in mg/dl

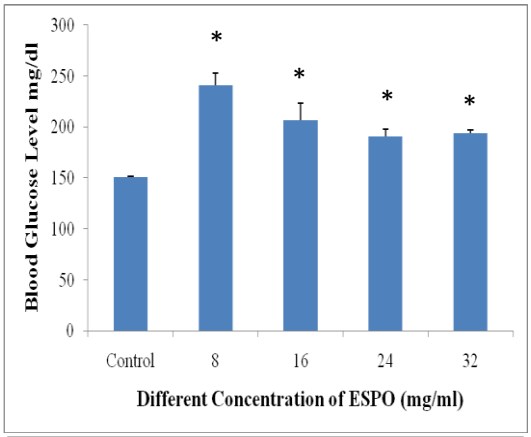

Fig 58 Diabetic mice treated by eri silkworm pupal oil and blood glucose level in mg/dl

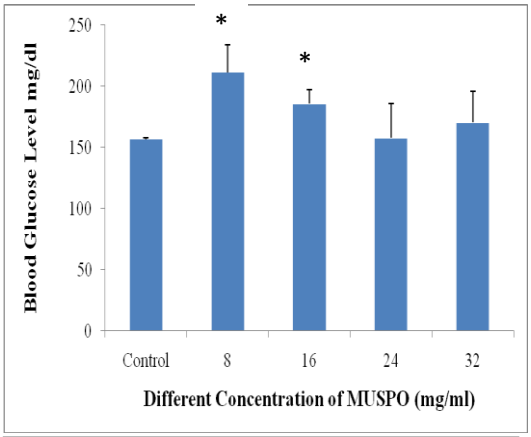

Fig 59. Diabetic mice treated by muga silkworm pupal oil and blood glucose level in mg/dl

4.6.1 Body weight

The body weight of diabetic rats showed a decrease compared with normal animals. The body weight of diabetic rats treated with mulberry and non-mulberry silkworm pupal oil significantly increased as compared with non-treated diabetic animals.

Table 21. Anti-Hyperlipidemic activity of mulberry and non-mulberry silkworm pupal oil by in vitro (organ bath) method:

Time in minutes	Mulberry pupal oil	Tasar pupal oil	Eri pupal oil	Muga pupal oil
0min	0.518±0.005	0.338±0.005	0.019±0.0001	0.165±0.006
15min	0.923±0.006	0.422±0.422	0.195±0.005	0.227±0.0051
30min	1.480±0.006	0.531±0.004	0.237±0.009	0.279±0.004
60min	2.080±0.005	1.234±0.001	0.335±0.009	0.547±0.004
120min	2.271±0.004	1.685±0.006	1.037±0.001	1.176±0.058
240min	2.462±0.001	1.894±0.001	1.041±0.004	1.214±0.062
480min	3.328±0.006	2.179±0.010	1.470±0.051	1.624±0.057
CV%	0.009	0.009	0.036	0.069
LSD $_{0.05}$	0.013	0.013	0.050	0.097
LSD $_{0.01}$	0.289	0.469	3.285	5.247

Data in Table 21 demonstrated that there are significant different ($P \leq 0.05$ %) in organ bath that the absorption of mulberry and non-mulberry (tasar, eri, muga) silkworm pupal oil in ileum (small intestine) of rat initially at 0min were 0.518±0.005, 0.338±0.005, 0.019±0.0001 and 0.165±0.006 respectively. The absorption of oil were increases with time interval increases as 15min, 30min, 60min, 120min, 240min and finally at 480min the absorption of mulberry and non-mulberry (tasar, eri, muga)

silkworm pupal oil were 3.328±0.006, 2.179±0.010, 1.470±0.051 and 1.624±0.057 respectively showed in table 21 and figure 60, 61, 62 and 63.

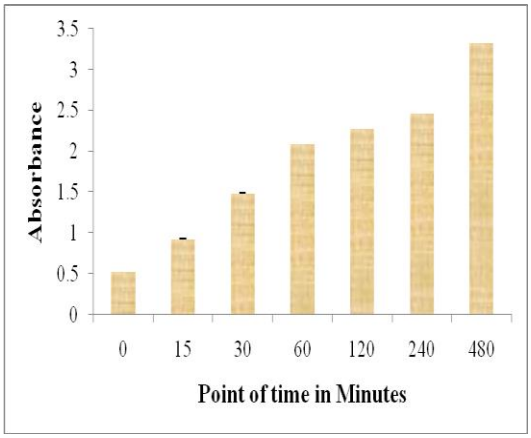

Fig 60. Absorption of mulberry silkworm pupal oil through ilieum of rat

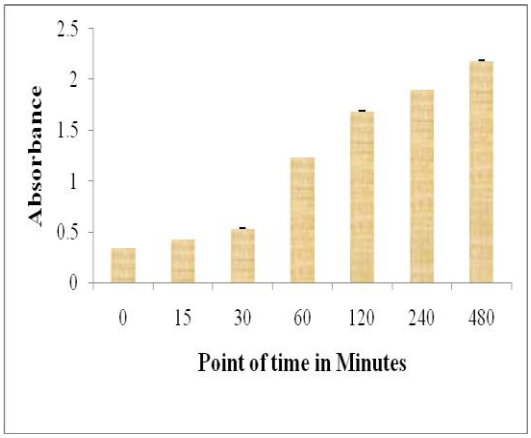

Fig 61. Absorption of tasar silkworm pupal oil through ilieum of rat

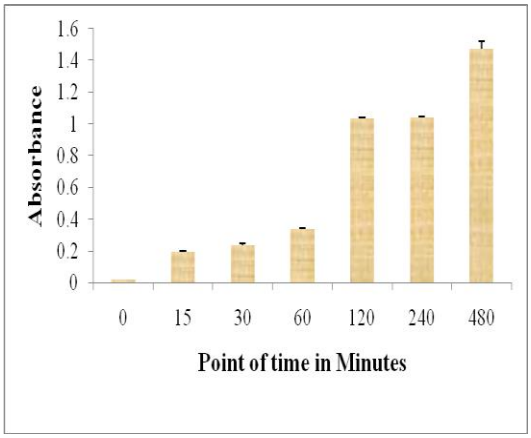

Fig 62. Absorption of eri silkworm pupal oil through ilieum of rat

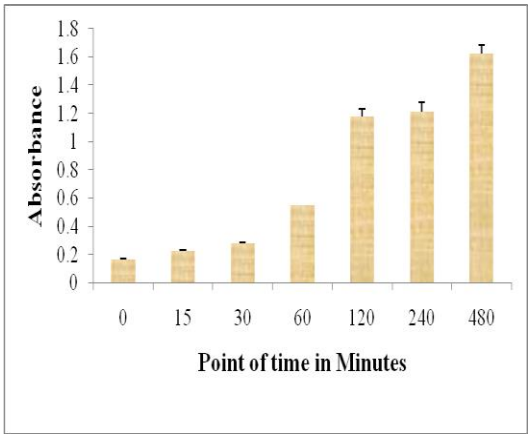

Fig 63. Absorption of muga silkworm pupal oil through ilieum of rat

4.6.2 Statistical Method (IBM SPSS)

Analysis of the data was of preventative variables in form mean ± SD. ANOVA (analysis of variance) used for comparison of *P* value (probability).

4.7 Discussion

Kumar *et al.*, (2012) studied on the investigation of antidiabetic, and hypolipidemic potential of *Cinnamomum tamala*, (Buch.-Ham.) Nees & Eberm (Tejpat) oil (CTO) in streptozotocin (STZ) induced diabetes in rats along with evaluation of chemical constituents. CTO (100 mg/kg and 200 mg/kg), cinnamaldehyde (20 mg/kg) and glibenclamide (0.6 mg/kg) in respective groups of diabetic animals administered for 28 days reduced the blood glucose level in Streptozotocin induced diabetic rats. There was significant increase in body weight, liver glycogen content, plasma insulin level and decrease in the blood glucose, glycosylated hemoglobin and total plasma cholesterol in test groups as compared to control group. In our studies results showed that after treatment of mulberry and non-mulberry (tasar, eri, muga) silkworm pupal oil there was a significant increase in body weight and reduction of blood glucose level.

The hypoglycemic properties of Shrimp (insect from Phylum Arthropoda) astaxanthin were studied by **Sila *et al.*, (2014)** on the kidney of alloxan-induced diabetic rats. For this experiment the animals were distributed into four groups of six rats each: a control group (C), a diabetic group (D), a diabetic group supplemented with Astaxanthin dissolved in olive oil and a diabetic group feedered with olive oil. In vitro antidiabetic cause was experimented in plasma and kidney tissue and found that group D of rats showed significant ($P \leq 0.05$) increase of glycemia, creatinine, urea and uric acid levels compared to those of the control group (C). Furthermore the plasma and kidney malondialdehyde and protein carbonyl levels for the rats of the group D were significantly amplified compared to the control group. Contrariwise, antioxidant enzyme activities, such as catalase, superoxide dismutase and non-enzymatic levels of reduced glutathione, were considerably ($P \leq 0.05$) decreases in the plasma and kidney of diabetic

rat's comparison shown by the control. The olive oil have omega-3 fatty acid and mulberry and non-mulberry silkworm pupal oil also having alpha-linolenic acid (omega-3) which further convert into EPA and DHA during metabolic activity. These compounds are helping to reduce blood glucose level.

Some researcher evaluates the antihyperglycemic and antihyperlipidemic effect of *L. usitatissimum* fixed oil. The oil (1, 2, 3 mL/kg) was investigated against Streptozotocin (STZ)-induced hyperglycemia and hyperlipidemia in albino rats, subjected to 3 weeks of treatment. The oil manifested decrease in blood glucose. The antihyperglycemic and antihyperlipidemic activity of the oil could be attributed to the presence of linolenic acid (ALA, 18:3 n-3) and its metabolic products, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) (**Kaithwas *et al.*,2012).** In our results mulberry and non-mulberry (tasar, eri, muga) silkworm pupal oil have linolenic acid which is a type of omega-3 fatty acid so, it also decrease the blood glucose level.

Several studies suggest that endocrine-metabolic diseases have close relation with the liver. Betatrophin is a type of hormone primarily expressed in liver and fat tissues. Yi *et al.* found that betatrophin can regulate metabolism by increasing insulin production via an increase in β cell mass **(Yi *et al.*, 2013)**

4.8 Conclusion

In conclusion, results of the present study demonstrate that oral administration of mulberry and non-mulberry (tasar, eri and muga) silkworm pupae oils and its main constituent has potential antidiabetic, antihyperlipidemic effect in STZ induced diabetic in rats in our model system. The previously discussed potent antioxidant activity of mulberry and non mulberry silkworm pupae oils may be responsible for the

antihyperlipidemic effects. This investigation reveals the potential of mulberry, tasar, eri and muga silkworm pupae oils for use as a natural agent with antidiabetic and antihyperlipidemic.

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Research Article Open Access

Analysis of Chemical Compounds in Different Mulberry and Non Mulberry Silkworm Pupae Powder by FTIR and EDX

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Abstract

Fourier transform infrared spectroscopy (FTIR) has become one of the states of art techniques to know the presence of potential functional groups in an analyte. In the present study the silkworm pupae powder of Mulberry, Tasar, Eri and Muga, before and after extraction of silkworm pupal oils were analyzed through FTIR spectroscopy to investigate different classes of compounds and their qualitative elemental composition. The FTIR spectra revealed the presence of different useful compounds like Alkanes, Alkenes, Alkynes, Aromatic compounds, Organic halogen compounds, Alcohols, Phenols, Ether Aldehydes, Esters, Carboxylic acid and Amides. The EDX analysis indicated that silkworm pupae powder both mulberry and non mulberry are rich in K (Potassium) and suggested that silkworm pupae waste can be effectively utilized as fertilizer for improving K content in soil, which play an important role in plant growth and development.

Keywords: Silkworm by-products; Organic compounds; Functional groups; Elemental analysis

Introduction

India is the world's second largest producer of raw silk after China. The rearing of silkworm is carried out throughout the year. Huge amount of pupal waste and silkworm litter are generated every year as a by-product of sericulture industry [1]. The silkworm pupae is one of the major by-products of silk industry; which has been considered as waste in silk reeling unit. Disposal of silkworm pupae meal has been a major problem encountered by sericulture industries across the world [2]. Such by-products which are presently discarded as waste can utilized for financial gains and generation of value-based products. The silkworm litter is presently used as fodder and compost and the pupal waste is also utilized in oil extraction, as a substrate in biogas production and mushroom cultivation [3]. The pupae of mulberry and non-mulberry silkworms have been extensively used as fertilizer, animal feed and edible insects in many countries, such as Japan, Korea, India and Thailand [4,5]. In the recent years, silkworm pupae have been incorporated in the list of "novel food resources managed as common food" by Ministry of Health P.R.China [6]. Further, some biologically active components in silkworm pupae have been widely explored for their enormous health benefits [7-10]. The pupa contains carbohydrate, protein, minerals, vitamins that are safe and vital to human body. After drying, it contains 54.54% protein, 32.7% saturated fatty acid and 67.3% unsaturated acid, linoleic acid of 6.94% and linolenic acid of 28.48%.

Several studies have revealed that the active substance in silkworm pupae is extracted by organic solvents and its free radical scavenging activity is used to determine the antioxidant property, to prevent aging by many oxidants [11]. The α-linolenic acid content has been reported to be as low as 0.6% in *Heliothis virescens* as high as 51% in *Hyalophora ceropia* silkworm pupae [12]. There are many reports to show that α-linolenic acid in the diet of animals can significantly reduce serum triglyceride levels [13]. Silkworm powder can be easily digested and absorbed by human bodies. It also can promote the physiological functions of the gastrointestinal tract. Furthermore, silkworm powder plays an excellent role in lowering blood glucose levels [14]. Silkworm pupae have much potential and many applications in pharmaceutical industry promoting human health. Silkworm pupae may contain several useful industrially important compounds which are required to be explored and characterized.

The present study was designed to evaluate the different classes of chemical compounds present in silk worm pupae powder which may be of industrial importance.

Experimental

Materials

Quality filter papers 11cm (110R) G-1, Petroleum Ether 40-60°C AR were purchased from Bionic Enterprises, Lucknow, Uttar Pradesh, India. The cocoons of three different silkworm varieties namely Tasar (*Antheraea mylitta*), Eri (*Philosamia ricini*), Muga (*Antheraea assamensis*) were collected from different places namely: Eri cocoons were taken from Banvasi Seva Ashram, Govindpur via Tura Sonbhadra, Uttar Pradesh. Tasar cocoons were obtained from Tasar silk Koya Market Dudhi, Pradesic Co-operative Sericulture Federation Limited Uttar Pradesh (Office of Assistant Director Sericulture Sonbhadra). Muga cocoons were taken from Central Muga Eri Research and Training Institute, Central Silk Board, Ministry of Textiles, Govt. of India, Assam. The mulberry silkworm (*Bombyx mori*) cocoons were produced at Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University by conducting silkworm rearing as per protocol suggested by Krishnaswami et al. [15].

Methods

Preparation of silkworm pupae powder: The pupae powder was

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prepared as per the protocol suggested by Wijaya singhe et al. [16]. After collection of mulberry and non mulberry (tasar, eri and muga) silkworm pupae, they were dried. The pupae were ground by using a mixer grinder to tear open the chitinous coating and to take out pupal matter. The chitinous matter was then manually separated from the pupal powder. The extraction of oil from the pupal powder was carried out by employing maceration method [17]. 10g dry pupae powder of the silkworm was transferred to a reagent bottle (100 ml). The solvent petroleum ether (30 ml) was added to the reagent bottle up to a level completely submerging the powder. The reagent bottle was closed tightly and sealed with glycerin to avoid evaporation. The reagent bottle kept for the period of 7 days. The contents of the regent bottle were filtered into a petri dish when the colour of the solvent turned yellow and the filtrate was kept in the open to evaporate the volatile solvent in which the oil was extracted. After extraction the pupal oil, the silkworm powder was used for experimental purpose.

FTIR analysis of silkworm pupae powder: The Fourier transforms infrared spectroscopy (FTIR) of four different silkworm types of pupae powder, before and after extraction of pupal oil were analyzed using Nicolet TM spectrometer (Thermo Scientific, USA). Pupal powder samples were embedded in KBr disc and 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 and all samples were performed in triplicates.

SEM-EDX analysis of silkworm pupae powder: The silkworm's pupae powder of mulberry and non-mulberry before and after extraction of oil were dried in oven at 60°C for four hours to remove moisture content. Dried samples were ground into fine powder using agate mortar. These samples were used for the SEM-EDX (Scanning Electron Microscope-Energy Dispersive Spectrometer) analysis. The microphotographs were recorded using SEM JEOL model, JSE-5610LV with an accelerating voltage of 20 keV, at high vacuum (HV) mode and Secondary Electron Image (SEI). The semi quantification elemental analysis to identify the weight percentage of major and minor elements present in the samples was done using the OXFORD INCA Energy Dispersive X-ray spectrometer (SEM-EDX).

Results and Discussion

In the present study, mulberry, tasar, eri and muga silkworm pupae powder (before and after extraction of silkworm pupa oil) were analyzed for the presence of different classes of potential chemical compounds and elemental composition.

IR analysis of mulberry, tasar, eri and muga silkworm pupae powder (before and after extraction of silkworm pupa oil).

FTIR analysis (Figure 1A-1H and Table 1) of mulberry silkworm pupae powder insinuated the presence of alkanes. The absorption arising from C-H region stretching in alkanes occurs in region between 3000-2840 cm^{-1}. In samples, MBSP-B, TSP-B, ESP-B, MUSP-B, MBSP-A, TSP-A, ESP-A and MUSP-A band occurred at 2855.2 cm^{-1}, 2854.8 cm^{-1}, 2855.2 cm^{-1}, 2855.4 cm^{-1}, 2855.2 cm^{-1}, 2855.4 cm^{-1}, 2854.8 cm^{-1} indicates C-H stretching in methyl group of alkanes. Bands at 2926.2 cm^{-1}, 2926.0 cm^{-1}, 2925.6 cm^{-1}, 2925.6 cm^{-1}, 2925.8 cm^{-1}, 2925.9 cm^{-1}, 2925.3 $cm^{-1}$and 2925.8 cm^{-1} occurred in all the samples before and after extraction of pupa oil represent asymmetrical stretching of CH_2 methylene group present in alkane (Table 1). Between the regions 1465-1150 cm^{-1}, the bands at 1458.9, 1455.6, 1456.4, 1406.4 , 1456.9, 1453.3, 1455.6, 1403.4 in case of the samples MBSP-B, TSP-B, ESP-B, MUSP-B, MBSP-A, TSP-A, and MUSP-A, C-H bending in methyl group of alkenes was observed (Table 1). The band occurred at 1458.9 cm^{-1} in MBSPB–B corresponding to C-H bending in alkanes. The isopropyl groups in alkane show a strong doublet, with peaks of almost equal intensities at 1385-1380 cm^{-1}. The tertiary butyl group in alkanes gives rise to C-H bending bands between the regions 1395-1385 cm^{-1} (Silverstein and Webster, 1998). In samples MBSP-B, TSP-B, ESP-B, MUSP-A, TSP-A, and ESP-A, the band at 1395.3 cm-^{1}, 1385.6, 1393.4 cm^{-1}, 1393.0 cm-^{1}, 1391.9 cm-^{1}, and 1397.8 cm^{-1} respectively are due to C-H bending (Table 1). Between the region 1350-1150 cm^{-1}, the bands at 1238.2 and 1158.0 cm^{-1} in MBSP-B (Figure 2 and Table 1) and the bands at 1237.6 and 1156.3 cm^{-1} in MBSP-A (Figure 1B and Table 1) were due to Methylene twisting and wagging vibrations. The bands assigned to C-C stretching vibrations are weak and appear in the broad region of 1200-800 cm^{-1} [18]. They are generally of little value of identification [18]. In case of both the sample the bands at 1082.2, 1118.4 and 1078.4 cm^{-1} indicated C-C stretching. Generally in C-H stretching between the regions 3100-3000 in alkenes results from aromatic C-H stretching [18]. In our results, the bands between this regions in all the samples except MBSP-A were occurred due to C-H stretching of aromatic ring. The C=C stretching mode of unconjugated alkenes usually shows moderate to weak absorption between 1667-1640 cm^{-1}. The bands at 1646.2 cm^{-1} and 1646.0 cm^{-1} in case of Muga silkworm powder before extraction and after extraction (MBSP-B and MBPB-A) 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 875.8 cm^{-1} in TSP-B, 880.7 cm^{-1} in MBSP-B, 877.2 cm^{-1} in TSP-A, 878.8 cm^{-1} in ESP-A and 880.0 cm^{-1} in MBSP-A may be due to out of plane C-H bending vibration of the vinyl, vinylididene group and transdisubstituted alkenes. Aromatic C-H stretching occur between the region 3100-2900 cm^{-1}. The band occurred at 3011.6 cm^{-1}, 3010.6 cm^{-1}, 3012.2 cm^{-1}, 3012.8 cm^{-1}, 3011.9 cm^{-1}, 3013.2 cm^{-1}, 3012.9 cm^{-1} and 2924.8 cm^{-1}in all the samples indicated C-H stretching (Table 1). The most prominent and most informative bands in the spectra of aromatic compounds occur in the low frequency range between 900-675 cm^{-1} [19]. These strong absorption bands result from the out of plane bending of the ring C-H bonds. Such bending was observed and recorded at band 623.2 cm^{-1} in TSP-B, at 626.1 cm^{-1} in MBSP-B, at 621.5 cm^{-1} in MBSP-A, at 623.9 cm^{-1} in TSP-A, at 622.7 cm^{-1} in ESP-A and at 625.4 cm^{-1} in MUSP-A. The aromatic C-H stretching and the skeletal vibrations absorb in the same regions as observed for the mononuclear aromatics. The most characteristics absorption of polynuclear aromatics results from C-H out of plane bending in region between 900-862 cm^{-1}. The band at 875.8 cm^{-1} in TSP-B, at 880.7 cm^{-1} in MBSP-B, at 877.2 cm^{-1} in TSP-A, at 878.8 cm^{-1} in ESP-A and at 880.0 cm^{-1} in MBSP-A revealed out of plane C-H bend of ring hydrogen. In the samples like MBSP-B, ESP-B and MBSP-A no such bands were observed (Figure 1A-1C and Table 1). Between the region 835-805 cm^{-1} a band was recorded at 832.9 cm^{-1} in MBSP-B indicating out of plane C-H bend of isolated Hydrogen. This band was not recorded in any sample. The band at 787.1 in TSP-B, 787.7 in ESP-A and MBSP-A were because of out of plane C-H bends of 2 adjacent H atom. The bands at 745.2 in TSP-B, 709.8 in ESP-B, 710.1 in TSP-A, 746.1 in ESP-A and 746.9, 708.7 in MBSP-A (Figure 1B-1F and Table 1) occurred due to out of plane C-H bend of 4 adjacent H atoms. The strong absorption of halogenated hydrocarbons arises from the stretching vibrations of the carbon halogen bond (Silverstein and Webster, 1998). Fluorine containing compounds absorb strongly over a wide range between

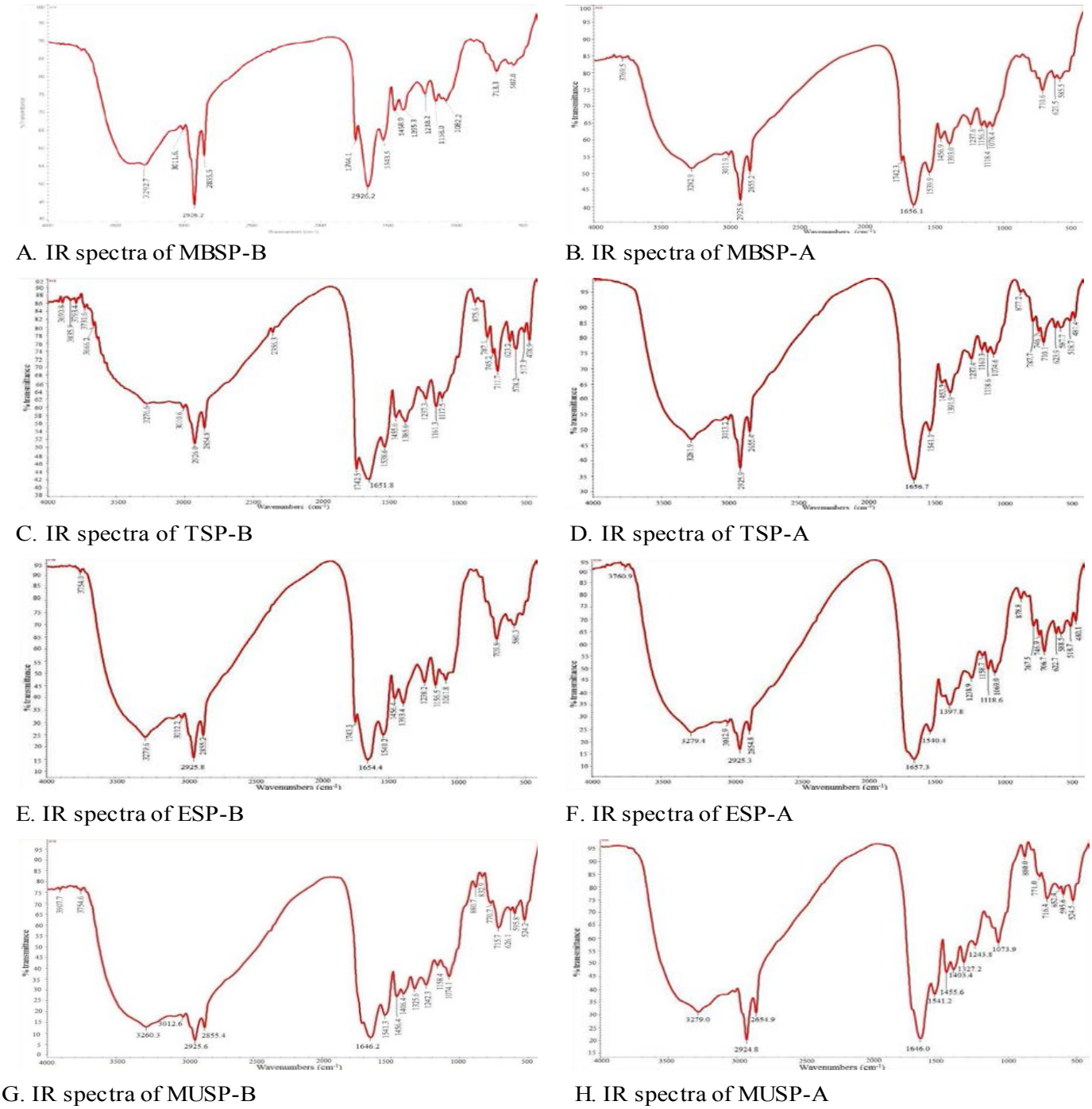
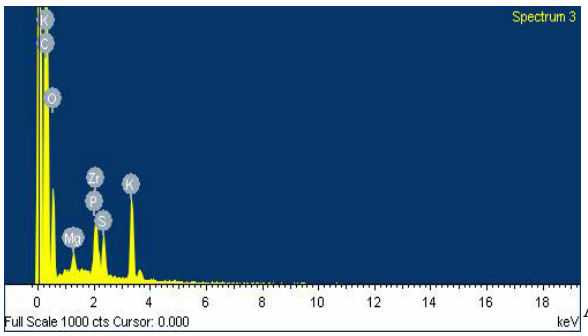


Figure 1: (A-H) EDX spectra of silkworm pupae powder. MBSP-B: Mulberry silkworm powder before extraction; TSP-B: Tasar silkworm powder before extraction; ESP-B: Eri silkworm powder before extraction; MUSP-B: Muga silkworm powder before extraction; MBSP-A: Mulberry silkworm powder after extraction; TSP-A: Tasar silkworm powder after extraction; ESP-A: Eri silkworm powder after extraction; MUSP-A: Muga silkworm powder After Extraction.

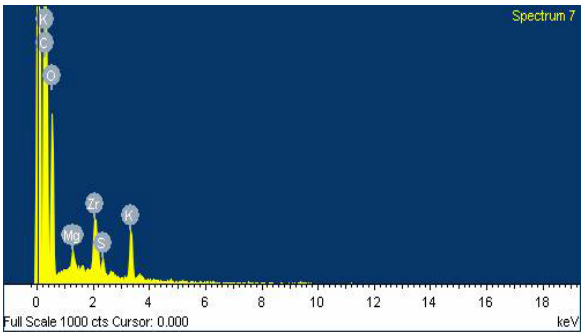
1400 and 1000 cm^{-1} because of C-F stretching modes. The C-F stretching was not observed in MBSP-A and ESP-B but in other samples *viz.,* MBSP-B, TSP-B, MUSP-B, ESP-A and TSP-A the bands at 1395.3 cm^{-1}, 1385.6 cm^{-1}, 1393.4 cm^{-1}, 1393.0 cm^{-1}, 1391.9 cm^{-1} and 1397.8 cm^{-1} indicated C-F stretching (Figure 1A; 1C; 1G; 1F, 1D and Table 1). The CF_3 and CF_2 groups in organic halogen compounds absorb mainly in the region between 1350-1100 cm^{-1}. A series of bands in this region were recorded in all the samples indicated CH_2 wagging in Cl, Br and I. Aliphatic C-Cl absorption is observed in the broad region between 800-600 cm^{-1}. Multiple bands between this region were recorded which indicate C-Cl stretching in all the samples analyzed. Brominated compounds absorb IR spectra in the region between 600-500 cm^{-1}(Silverstein and Webster, 1998). The bands at 587.8 cm^{-1}, 578.2 cm^{-1}, 580.3 cm^{-1}, 595.8 cm^{-1}, 585.5 cm^{-1}, 587.7 cm^{-1}, 588.5 cm^{-1} and 595.6 cm^{-1}

Frequency In cm^{-1}	MBSP-B	TSP-B	ESP-B	MUSP-B	MBSP-A	TSP-A	ESP-A	MUSP-A	Band Assignments
Alkanes									
3000-2840	2855.5	2854.8	2855.2	2855.4	2855.2	2855.4	2854.8	2854.9	C-H Stretching vibrations
2926-2853	2926.2	2926.0	2925.6	2925.6	2925.8	2925.9	2925.3	2924.8	Asymmetrical Stretching of CH_2
1465-1150	1458.9	1455.6	1456.4	1456.4 1406.4	1456.9	1453.3	-	1455.6 1403.4	Symmetrical C-H bending of CH_3
1395-1385	1395.3	1385.6	1393.4	-	1393.0	1391.9	1397.8	-	C-H bending
1350-1150	1238.2 1158.0	1237.3 1161.3	1238.2 1156.5	1242.3 1158.4	1237.6 1156.3	1237.4 1160.3	1238.9 1158.7	1243.8	Methyl twisting and wagging vibrations
1200-800	1082.2	1117.5 875.8	1081.6	1074.1 880.7 832.9	1118.4 1078.4	1118.6 1074.6 877.2	1118.6 1069.0 878.8	1073.9 880.0	C-C Stretching
Alkenes									
3100-3000	3011.6 2926.2	3010.6 2926.0	3012.2 2925.8	3012.8 2925.6	3011.9 2925.8	3013.2 2925.9	3012.9 2925.3	-	C-H stretching in aromatic alkene
1667-1640				1646.2				1646.0	C=C stretching vibration in unconjugated alkenes
1000-650	-	875.8	-	880.7	-	877.2	878.8	880.0	Out of plane C-H bending
Alkynes									
3100-2900	3011.6	3010.6	3012.2	3012.8	3011.9	3013.2	3012.9	2924.8	Alkyl C-H stretching
975-600	-	623.2	-	626.1	621.5	623.9	622.7	625.4	Out of plane C-H bending
Aromatic compounds									
900-862	-	875.8		880.7		877.2	878.8	880.0	Out of plane C-H bend of ring hydrogen
835-805	-	-	-	832.9	-	-	-	-	Out of plane C-H bend of isolated Hydrogen
810-785	-	787.1	-	-	-	-	787.7	787.7	Out of plane C-H bend of 2 adjacent H atom
760-600	-	745.2	709.8	-	-	710.1	746.1	746.9 708.7	Out of plane C-H bend of 4 adjacent H atom
Organic Halogen compounds									
1400-1100	1395.3	1385.6	1393.4		1393.0	1391.9	1397.8		C-F stretching
1350-1100	1238.2 1158.0	1237.3 1161.3 1117.5	1238.2 1156.5 1156.5	1325.6 1242.3 1158.4	1237.6 1156.3 1118.4	1237.4 1160.3 1118.6	1238.9 1158.7 1118.6	1327.2 1243.8	CH_2 wagging in Cl, Br and I
800-600	713.3	787.1 745.2 711.7 623.2	709.8	770.7 715.7 626.1	710.6 621.5	787.7 746.1 710.1 623.9	787.5 746.9 708.7 622.7	771.0 716.4 625.4	C-Cl stretching
600-500	587.8	578.2 517.3	580.3	595.8 524.2	585.5	587.7 518.7	588.5 518.7	595.6 524.5	C-Br stretching
Alcohols									
3700-3584	-	3666.2	-	-	-	-	-	-	O-H stretching vibration
3550-3200	3292.7	3276.6	3279.6	3280.3	3282.9	3281.9	3279.4	3279.0	O-H stretching vibration
2980-2840	2855.5	2854.8	2855.2	2855.4	2855.2	2855.4	2854.8	2854.9	C-H stretch: Methylene
Phenols									
1395-1360	1395.3	1385.6	1393.4	1325.6	1393.0	1391.9	1397.8	1327.2	In plane O-H bend
Ether									
1170-1114	-	1117.5	-	-	1118.4	1118.6	1118.6	-	Splitting of -C-O-C
1275-1200	1238.2	1237.3	1238.2	1242.3	1237.6	1237.4	1238.9	1243.8	-C-O-C stretching
Aldehyde									
2900-2820	2855..45	2854.8	2855.2	2855.4	2855.2	2855.4	2854.8	2854.9	C-H stretching
Esters									
1750-1735	1744.1	1742.5	1743.3	-	1742.3	-	-	-	C=O stretching
Carboxylic Acid									
3300-2500	2926.2	2926.0	2925.8	2925.6	2925.8	2925.9	2925.3	2924.8	O-H stretch
Amides									
1680-1630	1654.0	1651.8	1654.4	-	1656.1	1656.7	1657.3	1646.0	N-H bending
1650-1600	-	-	-	1646.2	-	-	-	-	C=O stretching
1570-1500	1543.5	1538.6	1540.2	1541.3	1539.9	1541.1	1540.4	1541.2	N-H bending

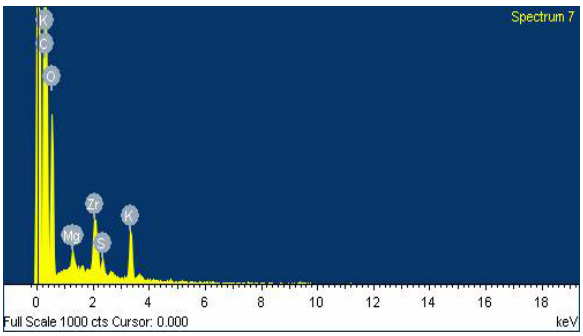
Table 1: Band assignments of FTIR Spectra of silkworm pupae powder. MBSP-B: Mulberry silkworm powder before extraction; TSP-B: Tasar silkworm powder before extraction; ESP-B: Eri silkworm powder before extraction; MUSP-B: Muga silkworm powder before extraction; MBSP-A: Mulberry silkworm powder after extraction; TSP-A: Tasar silkworm powder after extraction; ESP-A: Eri silkworm powder after extraction; MUSP-A: Muga silkworm powder After Extraction.



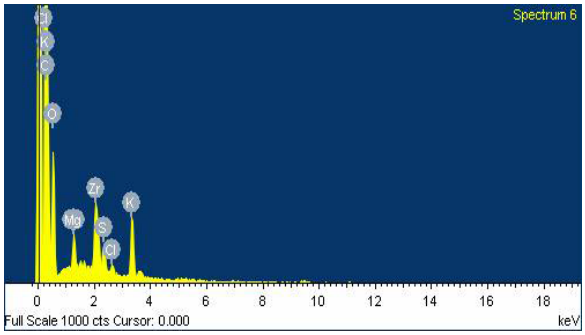
A. EDX spectrum of MBSP-B



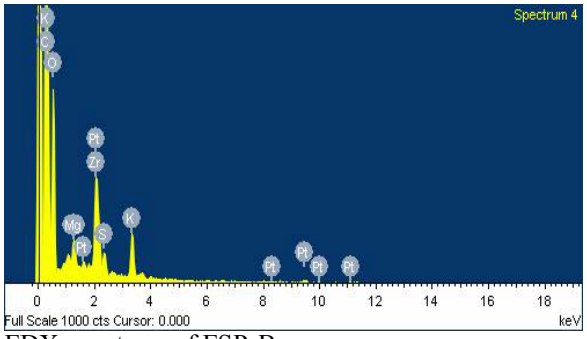
B. EDX spectrum of MBSP-A



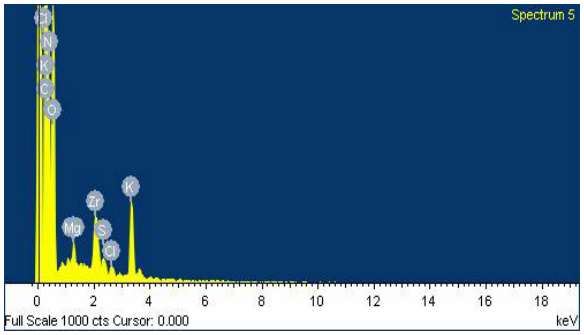
C. EDX spectrum of TSP-B



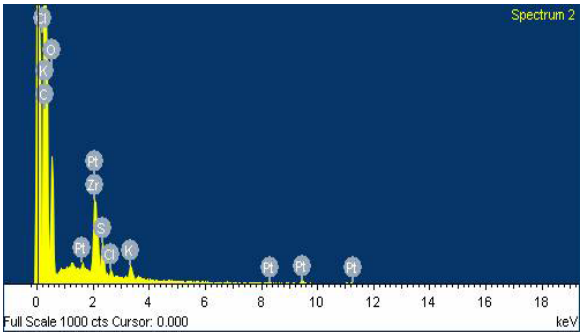
D. EDX spectrum of TSP-A



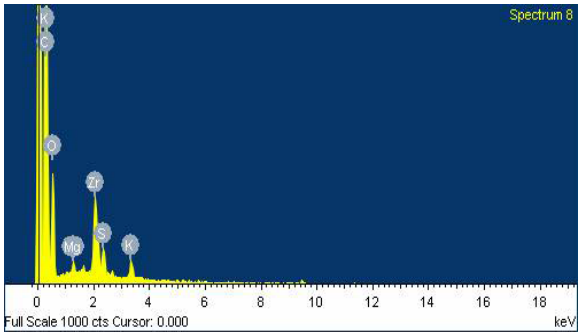
E. EDX spectrum of ESP-B



F. EDX spectrum of ESP-A



G. EDX spectrum of MUSP-B



H. EDX spectrum of MUSP-A

Figure 2: (A-H) FTIR spectra of silkworm pupae powder. MBSP-B: Mulberry silkworm powder before extraction; TSP-B: Tasar silkworm powder before extraction; ESP-B: Eri silkworm powder before extraction; MUSP-B: Muga silkworm powder before extraction; MBSP-A: Mulberry silkworm powder after extraction; TSP-A: Tasar silkworm powder after extraction; ESP-A: Eri silkworm powder after extraction; MUSP-A: Muga silkworm powder After Extraction.

in the samples *viz.*, MBSP-B, TSP-B, ESP-B, MUSP-B, MBSP-A, TSP-A, ESP-A and MUSP-A (Figure 1A-1H and Table 1) respectively indicate C-Br stretching. The non hydrogen bonded or free hydroxyl group of alcohols and phenols absorbs strongly in the region 3700-3584 cm^{-1} region. In TSP-B (Figure 1C and Table 1) the band at 3666.2 cm^{-1} indicated O-H stretching vibration in alcohols. However other

samples excluding TSP-B did not reveal this band. The bands at 3292.7, 3276.6 cm^{-1}, 3279.6 cm^{-1}, 3280.3 cm^{-1}, 3282.9 cm^{-1}, 3281.9 cm^{-1}, 3279.4 cm^{-1} and 3279.0 cm^{-1} in the samples *viz.,* MBSP-B, TSP-B, ESP-B, MUSP-B, MBSP-A, TSP-A, ESP-A and MUSP-A respectively correspond to O-H stretching vibration in alcohol. Between the region 2980-2840, the bands at 2855.5, 2854.8 cm^{-1}, 2855.2 cm^{-1}, 2855.4 cm^{-1}, 2855.2 cm^{-1}, 2855.4 cm^{-1}, 2854.8 cm^{-1}, 2854.9 cm^{-1}, in the samples *viz.,* MBSP-B, TSP-B, ESP-B, MBSP-B, MBSP-A, TSP-A, ESP-A and MUSP-A respectively (Figure 1A-1H and Table 1) correspond to C-H stretching in Methylene group. The bands between the regions 1395-1360 cm^{-1} correspond to in plane O-H bend in phenols. Several bands were recorded between these regions in the entire sample indicating presence of phenolic groups. The majority of aldehydes show aldehydic C-H stretching absorption between the regions 2900-2850 cm^{-1}. In all the samples bands between these particular regions were recorded conforming aldehydic C-H stretching. The C=O absorption band of saturated aliphatic esters is in the 1750-1735 cm^{-1} [18]. The bands appeared at 1744.1 cm^{-1}, 1742.5 cm^{-1}, 1743.3 cm^{-1} and 1742.3 cm^{-1} in the samples MBSP-B, TSP-B, ESP-B and MUSP-B (Figure 1A; 1D; 1E and 1G and Table 1) respectively may be due to C=O stretching of saturated aliphatic esters. In the samples like MUSP-B, TSP-A, ESP-A and MUSP-A these bands were not observed indicating absence of esters. Carboxylic acids show a strong, wide band for the O–H stretch. Unlike the O–H stretch band observed in alcohols, the carboxylic acid O–H stretch appears as a very broad band in the region 3300-2500 cm^{-1}, centered at about 3000 cm^{-1}. This is in the same region as the C–H stretching bands of both alkyl and aromatic groups. Thus a carboxylic acid shows a somewhat "messy" absorption pattern in the region 3300-2500 cm^{-1}, with the broad O–H band superimposed on the sharp C–H stretching bands. The reason that the O–H stretch band of carboxylic acids is so broad is because carboxylic acids usually exist as hydrogen-bonded dimers. In all the samples the bands were recorded between the region 3300-2500 indicating O-H stretching in carboxylic acid. Amides show a very strong C=O peak at 1680-1630 and N-H bending at 1640-1550 for both secondary and primary amides. In the samples like MBSP-B, TSP-B, MUSP-B, MUSP-A, TSP-A, ESP-A and MUSP-A the bands at 1654.0 cm^{-1}, 1651.8 cm^{-1}, 1654.4 cm^{-1}, 1656.1 cm^{-1}, 1656.1 cm^{-1}, 1656.7 cm^{-1}, 1657.3 cm^{-1} and 1646.0 cm-^{1} correspond to N-H bending in amides (Table 1). Such bands were not recorded in MSP-B indicating absence of Amide moieties. A band 1646.2 cm^{-1} between the region 1650-1600 was recorded in MUSP-B which was due to C=O stretching vibrations in primary amides. This band was not recorded in any other sample excluding MSPB. Secondary acyclic amides in the solid state display an amide II band in the region between 1550-1515 cm^{-1} [19]. In all the samples, bands between this regions indicated N-H bending in amides. IR analysis of different samples of silkworm pupae powder before and after extraction of oil revealed presence of different classes of chemical compounds namely Alkanes, Alkenes, Alkynes, Aromatic compounds, Organic Halogen compounds, Alcohols, Phenols, Ether Aldehyde, Esters, Carboxilic acid and Amides. Alkanes from silkworm pupae powder can be harvested and utilized as lubricants, LPG, automobile gasoline, aviation fuel [20]. Alkenes from the silkworm pupae powder can be harvested for their application in chemical industries for the preparation of polymer, glycols, aldehydes and other useful products. Isobutylene is used to make t-butanol. Alcohol is manufactured industrially from ethylene by hydration. Alkynes from the silkworm pupae powder can be harvested as raw material for textile fibers such as Orlon and Acrilon [20]. Aromatic hydrocarbons like tetralin and declin are used as solvent for paints and varnishes [20]. Different halogen compounds from silkworm pupae powder are very good solvent for non-polar compounds. Methylene chloride and chloroform dissolve old paints. Carban tetrachloride is used in dry cleaning as it dissolves oil and grease. Freon (CCl_2F_2), a fluorocarbon, is used as a refrigerant fluid. Some chemical agents like D.D.T., Aldrin, Chloradane, Dialdrin, Heptachlor, Lindane and Methoxychlor that kill and prevent the growth of pests [20]. Phenolic compounds from silkworm pupae powder can be harvested and utilized as antiseptic and disinfectants, as raw material for the manufacture of backelite and for the manufacture of several chemical such as phenolphthalein, picric acid and cyclohexanol. It is also used in manufacture of dyes and aspirin analgesic [20].

Esters from silkworm pupae powder can be used as artificial flavoring agent, in perfumes, as solvents for resins and as plasticizer. Certain esters such as ethyl p-dimethylaminobenzoate are constituents of sun-screen lotions and prevent the effect of UV rays. A series of compounds of carboxylic acids as citric acid esters, salicylic acid, mefenamic, anthranilic acid have diverse applications. Citric acid is used as plasticizers. Salol is used in coating pills in order to permit their contents to pass into the intestines. Aspirin is used widely because of its analgesic properties. Mefenamic acid, a derivative of anthranilic acid is used as an anti-inflammatory agent. Anthranilic acid is used in the preparation of indigo and in perfumery [20].

Bulk elemental analysis of silkworm pupae powder by EDX:

The semi quantification elemental analysis to identify the weight percentage of major and minor elements present in the samples was done using the OXFORD INCA Energy Dispersive X-ray spectrometer (SEM-EDX). This technique is being used in numerous applications for environmental science and technology. Qualitative elemental analysis of silkworm pupae powder was performed using SEM-EDX with a view to reveal in depth information on chemical speciation of silkworm pupae powder. The SEM-EDX analysis revealed that elements like K, C, O, Zn, Mg, P, S, Cl were present in all the samples. Element potassium was dominant element present in all the samples followed by carbon and oxygen. Potassium ions are an essential component of plant nutrition [21,22]. It is used as a fertilizer in agriculture, horticulture, and hydroponic culture in the form of chloride (KCl), sulfate (K_2SO_4), or nitrate (KNO_3). Agricultural fertilizers consume 95% of global potassium chemical production, and about 90% of this potassium is supplied as KCl [21,22]. The potassium cation is a nutrient necessary for human life and health. Potassium chloride is used as a substitute for table salt by those seeking to reduce sodium intake so as to control hypertension.

Conclusion

In present study an attempt has been made to investigate different chemical compounds in silkworm pupae powder using FTIR and EDX spectroscopy before and after extraction of pupal oil. The FTIR spectra insinuated the presence of different compounds like Alkanes, Alkenes, Alkynes, Aromatic compounds, Organic Halogen compounds, Alcohols, Phenols, Ether Aldehydes, Esters, Carboxylic acid and Amides useful for diverse applications. The EDX analysis revealed that silkworm pupae powder is rich in K and suggested that silkworm pupae waste can be effectively utilized as fertilizer for improving soil fertility even after extraction of pupal oil. The present study advocates the use of waste silkworm pupae powder as a feed stock for production of value added products for pharmaceutical and fertilizer industry. Employment of such feed stocks for extraction of useful chemicals offers a better alternative for disposal and/or recycling wastes and minimizes environmental pollution. Future prospects in this context shall include cost effective extraction technologies and development of industrial applications.

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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

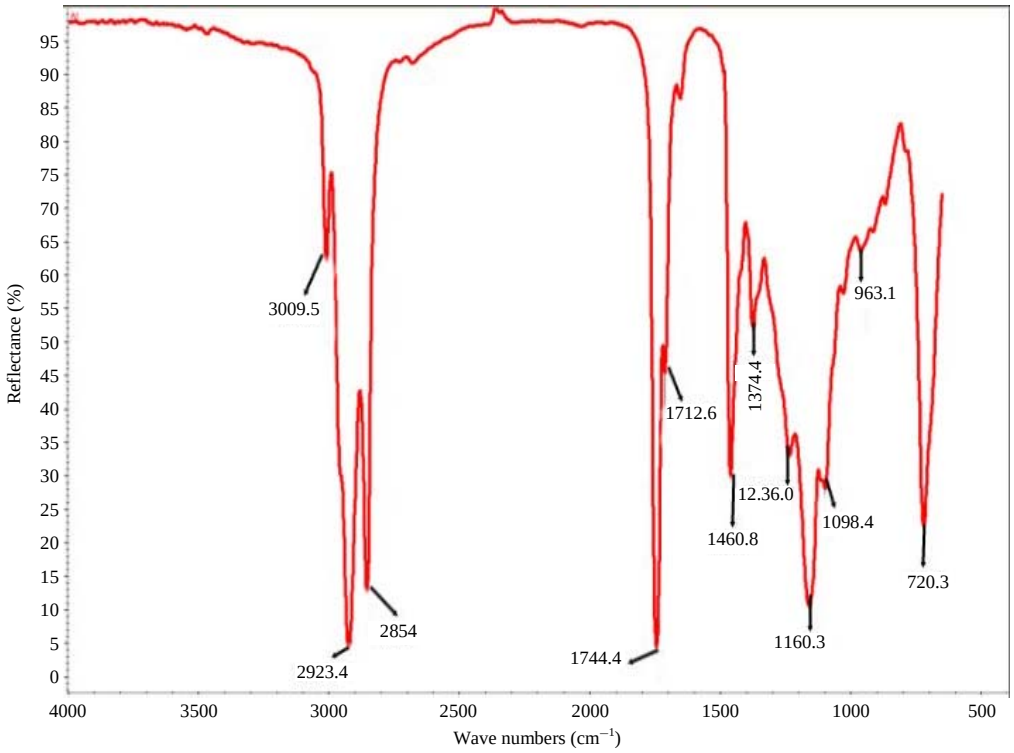

Fig. 1: FT IR spectrum of Mulberry silkworm pupal oil

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

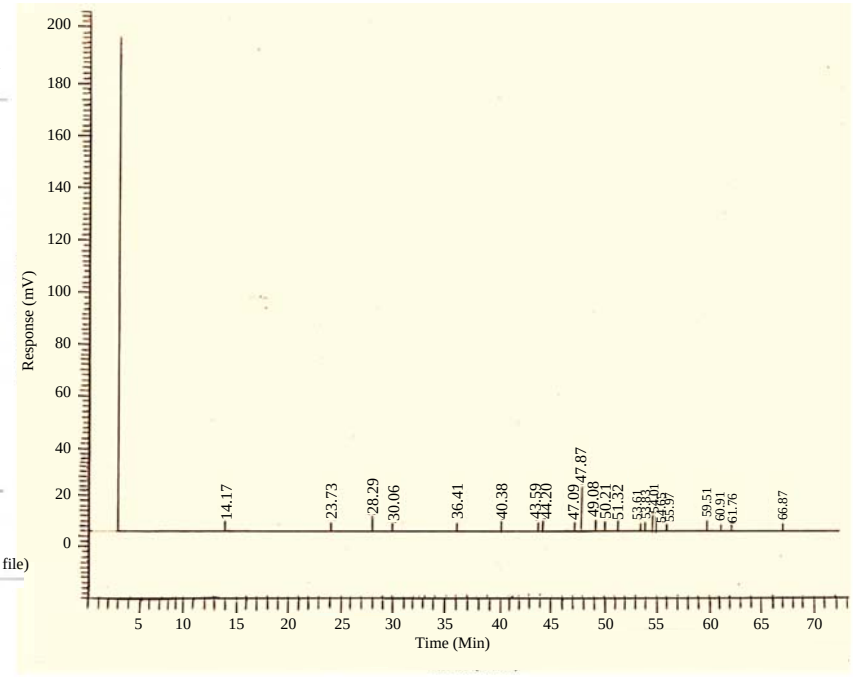

Fig. 2: Retention time of mulberry silkworm pupal oil and GC-MS spectra of mulberry silkworm pupal oil

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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Biomedical Applications of Silkworm Pupae Proteins

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Chapter 3
Biomedical Applications of Silkworm Pupae Proteins

Dhiraj Kumar, Param Dev and R.Venkatesh Kumar

Abstract Silkworm is a biologically important and unique insect which engineer a structure called cocoon. Apart from extraction of silk fiber from cocoons, this complex fibrous protein membranous shell ensures the successful metamorphosis of the silkworm larvae to pupae and finally to silk moth. The pupae of mulberry and non-mulberry silkworms have been in consideration as new available source of high quality protein that contains all the essential amino acids required for human health. In recent years, research has been focused on various biomedical applications of silkworm pupae proteins. Pupae proteins are efficiently worked in wound dressings, hepatoprotective and antiapoptotic activity, antigenotoxicity, regulation of blood glucose and lipids, anticancer agent, etc. Therefore, silkworm pupae could be utilized as food supplement and its enormous proteins open the new dimension for biomedical science.

Keywords Silkworm pupae · Proteins · Diseases · Biomedical applications

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3.1 Introduction

Among the economically beneficial insects, silkworms are an incredibly significant animal model for researchers next to Drosophila, belonging to the order Lepidoptera of phylum Arthropoda. India is the only country which hosts all five known commercially exploited types of natural silkworms (Mulberry, tropical tasar, temperate tasar, eri, and muga) along with several of their wild relatives. During metamorphosis all five types of silkworms pass from egg, larva, pupa, and adult stages to complete their life cycle, therefore they are known as holometabolic insects. Silkworms are the only identified insects that provide food, fiber, and biomedical significance. After spinning of silk (cocoon) at larval stage they convert into pupa inside the cocoon shell. Silkworm pupae are obtained after the extraction procedure of silk thread and are not used commercially as an edible insect in India except in the northeastern states. They consume silkworm pupa in their diet regularly due to its rich protein content and several medicinal properties.

Silkworm pupae protein has been considered to be a new available source of high-quality protein that contains all the amino acids needed by the human body. Nevertheless, it is not popular among consumers, where silkworm pupae are an interesting optional product. Silkworm pupae are used as animal feed, organic fertilizer, food material, and traditional medicine in a few Asian countries, namely Korea, China, Thailand, Japan, and India [1–5]. Silkworm pupae are the optional food product and people began its commercialization in different food and biomedical industry to fulfill the nutritional requirements of hidden hungers and treatment of various diseases of mankind.

3.2 Nutritional Value of Silkworm Pupae

Silkworm pupae contain 55.60 % of total protein and 32.2 % lipid content by dry weight. The silkworm pupae protein is boosted with high level of essential amino acids, namely methionine, valine, and phenylalanine. The contents of essential amino acids of silkworm pupae protein fulfill the requirements of FAO/WHO/UNU as they suggested in 2007. Aside from the high protein content it is also a chief source of omega-3 fatty acids, particularly α-linolenic, linoleic acid, DHA, and EPA.

Silkworm pupae per 100 g sample contain various biochemicals such as 55 g protein, 8.5 g fat, 6 g fiber, 25.43 g carbohydrates, and 389.60 (Kcal/100 g) energy contents. Silkworm pupae also contain diverse mineral compositions (mg/100 g) such as 102.31 mg calcium, 1826.59 mg potassium, 287.96 mg magnesium, 1369.94 mg phosphorus, 274.57 mg sodium, 9.54 mg iron, 17.75 mg zinc, 2.08 mg manganese and copper, and 0.08 mg selenium to complete the mineral requirement in a healthy diet. Additionally, it also comprises a number of vitamins such as Vitamin A (273.99 μg), Vitamin E (51.45 IU/kg), Vitamin C (<5.78 mg), Vitamin B1 (1.91 mg), Vitamin B2 (5.43 mg), Vitamin B3 (15.20 mg), Vitamin B5 (12.49 mg), Vitamin B7 (144.51 μg), Vitamin B9 (0.41 mg), and Vitamin B12 (0.5 mg/100 g).

Table 3.1 Amino acid composition in different types of silkworm pupae

Amino acid composition (g/100 g)	Mulberry silkworm pupae	Tasar silkworm pupae	Eri silkworm pupae
Aspartic acid	10.9	6.41	9.89
Threonine	5.4	4.64	4.75
Serine	4.7	4.64	5.25
Glutamic acid	14.9	12.7	12.9
Proline	4.0	–	6.46
Glycine	4.6	4.42	4.94
Alanine	5.5	6.26	6.14
Cystine	1.4	1.5	0.53
Valine	5.6	6.63	5.36
Methionine	4.6	1.47	2.31
Isoleucine	5.7	7.95	4.42
Leucine	8.3	3.24	6.63
Tyrosine	5.4	2.06	6.40
Phenylalanine	5.1	8.1	5.24
Histidine	2.5	2.94	2.67
Lysine	7.5	4.54	6.54
Arginine	6.8	12.2	4.41
Total amino acids	102	89.8	94.8
Total essential amino acids	51.5	43.1	44.9

Source Roa [8], Zhou et al. [9, 10], Longvah et al. [11]

In the fat bodies of larvae, pupae, and moths of silkworm 138, 217 and 86 expression of protein profiles were determined, respectively, of which 12 were shared by the three stages. 92, 150, and 45 specific proteins were identified in the larval, pupal, and moth stages, respectively, in which 17, 68, and 9 had very important functional annotations. Numerous ribosomal proteins (L4, L5, L23, P2, S3, S10, S11, and S15A) are established in fat bodies of silkworm pupae, while only three (L14, S7, and S20) were found in larval and moth fat bodies. Furthermore, 23 metabolic enzymes are also present in the pupal stage of silkworm, whereas only four and two metabolic enzymes are known in the larval and moth stages [6]. Recently, it has been documented that silkworm pupae have excellent antioxidant potential to scavenge free radicals and good antityrosinase activity and also high levels of palmitic acid, oleic acid, stearic acid, linoleic acid, and palmitoleic acid in profiles of fatty acids [7]. Amino acids composition in different types of silkworms pupae are shown in Table 3.1.

3.3 Biomedical Applications of Silkworm Pupae and Their Protein

3.3.1 Antiapoptotic Activity of 30 kDa Lipoproteins

Silkworm pupa fat body and hemolymph are abundant in 30 kDa family of lipoproteins (LP1-5) and are low molecular weight lipoproteins. It was confirmed that the silkworm protein 30Kc6 is one of the members of the 30K family proteins that transport lipids and inhibit cell apoptosis in the insect and mammalian cells [12–15]. However, effects of 30Kc6 on cell apoptosis of human vascular endothelial cell (HUVEC) and the underlying mechanism are largely unknown.

In vivo data of Wei [16] demonstrated that oral feeding of the silkworm protein 30Kc6 dramatically improved the conditions of atherosclerotic rabbits by decreasing serum levels of total triglyceride (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and total cholesterol (TC). Furthermore, 30Kc6 alleviated the extent of lesions in aorta and liver in atherosclerotic rabbits. These data are not only helpful in understanding the antiapoptotic mechanism of the 30K family proteins, but also provide important information about prevention and treatment of human cardiovascular diseases.

3.3.2 Antioxidant, Antigenotoxic, and Hepatoprotective Properties

Silkworm pupae contain vitamin B2, which can be important to avoid the serious effects of vitamin B2 deficiency [17]. Further, fermented silkworm powder has a protective effect in alcohol-induced hepatotoxicity in a rat model [18]. Further, it is reported that silkworm pupae contain 45–55 % protein content on a dry matter basis, which can significantly increase the hemoglobin and serum total protein in rats, producing protective effects on the liver in carbon tetrachloride-induced rat hepatic injury [19, 20]. Meetali [7] also evaluated the antioxidant and antigenotoxic effects of Muga silkworm pupa, therefore, it is recommended that silkworm pupae can be utilized as natural antioxidants in various food products.

3.3.3 Role as Bioreactor

Researchers report that recombinant proteins have been identified in *Bombyx mori* cells or silkworm larvae, apart from pupae. Alternatively, Jian [21] selected silkworm pupae to express the protein of interest using *B. mori* nucleopolyhedrovirus (BmNPV). They studied the expression, purification, and characterization of human granulocyte macrophage colony stimulating factor (GM-CSF) using silkworm pupae (*B. mori*) and concluded that silkworm pupae could be more suitable for expression of heterologous proteins as a bioreactor.

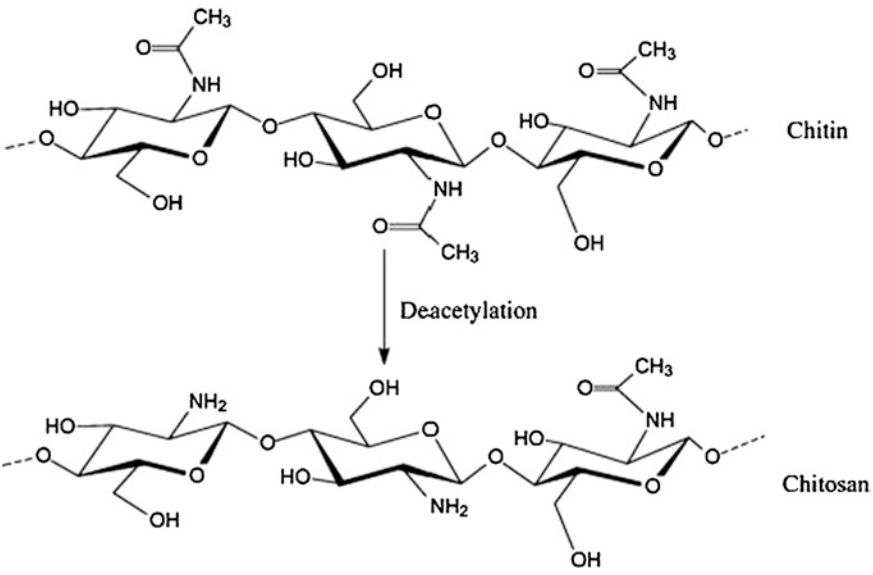

Fig. 3.1 Mechanism of chitosan production from chitin

3.3.4 Silkworm Pupae Chrysalides and Its Role in Pharmaceutical Industries

Silkworm pupae exoskeleton and internal organs such as spiracle and tracheae are lined by chitin. Nevertheless, the chrysalides of the silkworm pupae are an alternative source of chitin and consequently of chitosan [22].

β-1,4-N-acetyl-D-glucosamine (chitosan) is a derivative of chitin after deacetylation (Fig. 3.1). Importantly, chitosan is a biodegradable cationic biopolymer and could assist in reduction of pollutants in residual waters by adsorption and chelating with heavy metallic ions and can also act in coagulation of colloidal particles and silkworm pupae chrysalides having anticancerous property [23–26]. The fatty acids of odd numbers contained in the silkworm chrysalis oil also has high antitumor activity. Despite this, chitin and chitosan are being exploited in a variety of biomedical applications, including drug delivery, tissue engineering, tissue scaffolds, and wound dressings. The polycationic properties of chitosan are being developed for use in biosensors by immobilizing enzymes, in wound dressings to induce cell migration and proliferation at the wound site, and in tissue engineering as a scaffold [27].

Chitin has a great role in silkworm. Chitin has been used to prepare affinity chromatography column to isolate lectins and determine their structure [28]. Chitin and 6-O-carboxymethyl chitin activate peritoneal macrophages in vivo, suppress the growth of tumor cells in mice, and stimulate nonspecific host resistance against *Escherichia coli* infection.

Marguerite [29] studied the possible applications of chitin and chitosan. Austin [30] and Hirano [31] for the first time processed chitin in the form of films and fibers from silkworm pupae skin. However, the major development of chitin film and fiber is in pharmaceutical and medical applications as wound dressing material [32, 33] and controlled drug release [34]. In addition, an interesting application is composite bone filling material, which forms a self-hardening paste for tissue regeneration in treatment of periodontal bony defects [35] and its oligomers have been claimed as anticancer drugs. Wattanathron [36] identified that silkworm pupae protect against Alzheimer's disease. Biological properties of chitosan are biocompatibility, hemostatic, bacteriostatic, fungistatic, spermicidal, and anticholestermis.

3.4 Other Significant Biomedical Applications of Silkworm Pupae

Oiled and de-oiled silkworm pupae contain high-quality and quantity of protein. There are various pharmacological functions in the human body also recorded.

3.4.1 Regulation of Blood Lipids

Pupal oil can effectively reduce triglycerides, prevent and treat fatty livers [37], protect the liver after consumption of alcohols, improve the blood quality and the environment within the blood vessel, effectively soften the blood vessels, lower blood pressure, and prevent arteriosclerosis and thrombosis.

3.4.2 Reduction of Blood Glucose

Pupal oil enables the prostaglandins to maintain balance with effects of preventing prostate diseases, improving the functions of insulin-producing beta cells, restoring the fatty acid desaturase activity of cells in diabetic patients and has marked hypoglycemic effect free from reoccurrence [38].

3.4.3 Improvement of Physical Fitness

The natural steroids contained in the oil can improve fertility and enhance sexual function; the unsaturated fatty acids in the oil which cannot be synthesized by

humans can enhance the flexibility of immune cell membrane, increase the vitality of the immune cells, so that the barriers to human health are more robust, and the occurrence of sub-health and disease is effectively prevented.

3.4.4 Replenishment of Brain Power and Enhancement of Intelligence

The metabolites EPA and DHA, commonly known as Brain Gold, can promote the synthesis of nucleic acid and monoamine neurotransmitters in the brain and effectively enhance the mental memory.

3.4.5 Skin Care

The α-linolenic acid and other active substances contained in pupal oil can join the synthesis of human tissue cell membranes, effectively prevent and mitigate symptoms like wrinkles, pigmentation, sallow skins, and premature aging of modern women. At the same time it can improve the body superoxide dismutase activity and decrease free radical with good antiaging effects.

3.5 Future Prospective and Conclusion

Silkworm pupae are natural enormous byproducts of the silk industry with excellent applications in the field of biomedical science and the pharmaceutical industry. The proteins extracted or identified from silkworm pupae expressed great medicinal value to cure different deadly diseases. Most importantly, functions of pupal protein include drug delivery, tissue engineering, tissue scaffolds, wound dressings, regulation of blood lipids and glucose, antiapoptotic, antioxidant, antigenotoxic, hepatoprotective activity, bioreactor, and as anticancer agent.

Silkworms can be used as future animal models since they have been exploited for commercial importance in the past few years for production of recombinant proteins. Concomitantly, the above study will generate valuable information about proteomics of silkworm pupae and in future potential pupal proteins should be investigated through metagenomics, which could be exploited commercially by biomedical, pharmaceutical, biotech, and probiotic industries for the benefit of human health.

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FTIR and GC/MS Analysis of *Antheraea mylitta* Silkworm Pupal Oil and Its Antibacterial Activity Against *Staphylococcus sciuri* Strain CD97

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ABSTRACT

The present study investigated the chemical composition and antibacterial activity of *Antheraea mylitta* (Tasar) silkworm pupal oil.The chemical composition was analyzed with Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography Mass Spectrophotometry (GC/MS). 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 andamides while,GC-MS analysis revealed the existence of the Stearic acid, Palmitic acid and Linoleic acid. The antibacterialtest revealed high antagonistic potentiality of *A. mylitta* pupal oil against *Staphylococcus sciuri* strain CD97 at 100 and 110 µl/ml concentrations.

Key words *Antibacterial activity; FTIR; GC/MS; MIC; Tasar Silkworm pupal oil*

China people rear the oak Tasarsilk worm for ingestion and nowdays its pupae have also became an innovative food resources (Zhou and Han, 2006). Untilnow, very rare information is available regarding nutritional composition of oak silkworm *Antheraea pernyi.* Recently, extraction of oak silkworm pupal oil by supercritical carbon dioxide (SC-CO_2) has become violently used in various fields, such as food science, natural products, bioactive compounds, by-product recovery and the pharmaceutical and environmental sciences (Herrero *et al.,* 2009, Wang *et al.,* 2011).

Separation of â-sitosterol, oils or some exclusive fatty acids (e.g., gamma-linolenic, polyunsaturated fatty acid)can also be done by SC-CO_2 extraction (Sajfrtova *et al.,* 2010). Supercritical carbon dioxide (SC-CO_2) extraction has several advantages, including non-explosive, nontoxic and non-solvent residues, high purity and low cost (Sajfrtova *et al.,* 2010, Vidovic *et al.,* 2011, Herrero *et al.,* 2006). SC-CO_2 systems can be operated at low temperature, which avoid degradation allied with heat induction (Krichnavaruk *et al.,* 2008). Response surface methodology (RSM) is an effective and powerful statistical method for optimizing experimental conditions and the examination of severe processes with a reduced number of experimental trials (Wei *et al.,* 2009). From the past several years, RSM has been working successfully to optimize the supercritical CO_2 extraction of Cotton seed oil (Bhattacharjee *et al.,* 2007), Soybean oil (Jokic *et al.,* 2010), Pomegranate seed oil (Liu *et al.,* 2009), Rosehip seed oil (Machmudah *et al.,* 2007), Pumpkin seed oil (Mitra *et al.,* 2009] and Tea seedoil (Wang *et al.,* 2011).

Silkworm pupae oils are derivative of lipids, so it's constituent are very complex therefore, aproper method is necessary to analyze their contents by converting both fatty acid salts and the acyl components in all lipid classes, such as triacylglycerols, phospholipids, sphingolipids, and waxes, to methylesters using an efficient estrification process. O'Fallon*et al,*(2007) presented a method to openly methylate fatty acids from muscle tissue, oils, andfeedstuffs in aqueous solution that is based on amazing thought, the adding of water to the fatty acid methyl ester synthesis reagents. Silkworm pupae oil contains various essential fatty acids with bioactivity and thus can be used as raw material for cosmetics. Pupae oil is used in the cosmetic industries for making soaps and moisturizers (Kotake-Nara *et a*l., 2002).

Fatty acids functions as a key ingredient of antimicrobial food additives due to their inhibitory action on undesirable microorganisms (Freese, *et al.,* 1973). Additionally, the long-chain unsaturated fatty acids such as linoleic acid,Linolenic acid(LNA) and oleic acids are bactericidal to some important pathogenic microorganisms, including methicillin-resistant *Staphylococcus aureus* (Farrington, *et al.,* 1992; Kabara, *et al.,* 1972; Knapp and Melly, 1986), *Helicobacter pylori* (Hazell and Graham, 1990; Sun, *et al.,* 2003) and Mycobacteria (Seidel and Taylor, 2004).

MATERIALS AND METHODS

Pupae PowderPreparation

Pupae of the Tasar silkworm *Antheraea mylitta* cocoons were obtained from Tasar silk Koya Market Dudhi, Pradesic Co-operative Sericulture Federation Limited Uttar Pradesh (Office of Assistant Director Sericulture Sonbhadra). The pupae powder was prepared as the protocol suggested by Wijaya Singh *et al,*. (1977). For sample preparation, the collected pupae were dried and 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 silkworm pupal oil

The extraction of Tasarsilk worm pupae oil was done by following maceration method. For extract preparation, two different solvents were used i.e. petroleum and ether respectively. About 100g of Tasar silkworm pupal powder

Table 1. The characteristic absorption frequencies of functional groups for Tasar silkworm pupal oil

Functional group	Vibration and intensity	Frequency in Cm^{-1}	Peak Value
Alkanes	C-H str; m, s	2960-2850	2923.5
			2853.5
	C-H bend; m	1485-1440	1463.2
	C-H bend; m	1470-1430	1463.2
	C-H bend; w	1485-1340	1463.2
			1417.9
	C-C str; w	1300-800	1377
			1237.2
			1160.3
			1099.6
			1068.6
			967.2
			915
			866.6
Alkenes	C=C-H str; m	3100-3000	3010.8
	C-H; m	3040-3010	3010.8
	C-H bend; s	970-960	967.2
	C-H bend; s	915-905	915.0
Alkynes and Cycloalkanes	C-H str; m	3100-2920	2923.5
			3010.8
Aromatic Compound	Ar-H str; v	3050-3000	3010.8
	C-H bend; s	900-700	866.6
			792.3
			720.8
Halogen Compound	C-F str; s	1400-1000	1377.0
			1237.2
			1160.3
			1099.6
	C-Cl str; s		1068.6
		800-600	792.3
			720.8
Alcohols	O-H str; v, sh	3700-3500	3628.7
	O-H str; v, sh	3570-3450	3467.5
	O-H str; s	3000-2500	2923.5
			2853.5
Phenols	C-O str; s	1400-1310	1377.0
	C-O str;s	1410-1300	1377.0
Ether	C-O str; s	1270-1200	1237.2
	C-O str; s	1150-1070	1099.6
	C-O str; s	~910	-
Aldehydes	C-H str; w	2900-2820	2853.5
Ketones	C=O str; s	~1745	1743.5
	C=O str; s	1725-1710	-
	C=O str; s	1725-1700	-
	C=O str; s	1715-1690	-
Esters	C=O str; s	1750-1735	1743.5
	C=O str; s	1730-1715	-
Lactones	C=O str; s	1760-1740	1743.5
	C=O str; s	1750-1735	1743.5
		1715-1694	-
		1750-1735	1743.5
Saturated Aliphatic Acids	C=O str; s	1725-1700	-
	C=O str; s	1715-1694	-
Carboxylic Acid	O-H str; w, b	3000-2500	2923.5
			2853.5
Acid Anhydrides	C=O str; s	1790-1740	1743.5
	C=O str; s	1770-1725	1743.5
Lactams	C=O str; s	1760-1730	1743.5
Amines	N-H str; m	3500-3300	3467.5
Nitro Compound	N=O str; s	1375-1275	-

was added to 150ml of both the solvents separately in a reagent bottle. The reagent bottles were closed tightly and sealed with glycerin to avoid evaporation. The contents of the reagent bottles were filtered using filter paper, into petri dish after 7 day when the colour of the solvent changed to yellow. The filtrates were retained in the open in shed room to evaporate all volatile solvent in which the oil was extracted (Nipha and Arunyakorn, 1997). The extracted pupal oil was then used for further experimental purposes.

FTIR Analysis of Tasar silkworm pupal oil

Tasar silkworm pupae oil was analyzed using Nicolet TM spectrometer (Thermo Scientific, USA). The samples of pupal oil 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 and all samples were performed in triplicates.

GC/MS Analysis of Tasar silkworm pupal oil

The methylation of Tasar silkworm pupal oil was done by H_2SO_4–MeOH methylation method (Hammond, 1993). For this, the samplewere prepared by mixing 1g of Tasar silkworm pupal oil into 10 mL of H_2SO_4/MeOH/Toluene (1:10:20) reagent and refluxed on a water bath for 1 hour. The reaction mixture was diluted with 1mL 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 (30m x 0.25mm x 0.25µm). The oven temperature program of GC-MS was as follows: 70%C (3^0C/min)250^{0}C (6^0/min) 290^{0}C - 60^{0}C (3^0C/min) 220^{0}C (7^0C). Helium (He, 1mL/min) was used as a carrier gas, split ratio was 1:90, injecting temperature 250^{0}C in GC-MS, and the

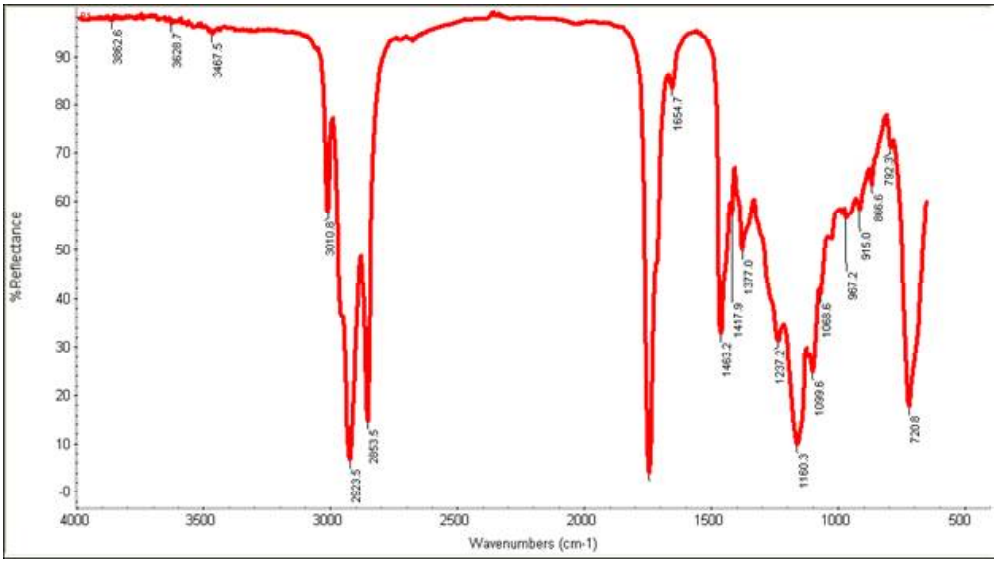

Fig.1. FT-IR spectrum of Tasar silkworm pupal oil.

290^{0}Cin split ratio 1:30, detector at 300^{0}C, hydrogen use as carrier gas 10Psi in GC.

Minimum Inhibitory Concentration (MIC) Test

The MIC test of Tasar silkworm pupal oil against *Staphylococcus sciuri* strain CD97was done by using Fuente *et al.,* (2006)method. The experimentwas conducted in five test tubes having different treatments i.e. First test tube is control, as media with bacteria and fouras antibiotic streptomycin at different concentration of 12.5, 25, 50, 100µl respectively. Another five test tubes were taken in which

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 3:26:29 PM
Data Acquisition Time : 3/3/15 1:40:15 PM
Channel : A
Operator : manager
Dilution Factor : 1.000000

Result File : C:\PenExe\TcWS\Ver6.2.0\Examples\March_2015\LNMB1_030315_-20150303-145603.rst
Sequence File : C:\PenExe\TcWS\Ver6.2.0\Examples\LNMB1_030315.seq

CIMAP

Peak #	Component Name	Time [min]	Area [uV*sec]	Area [%]
1		14.204	1513.33	0.10
2		28.385	1118.18	0.08
3		30.103	2793.84	0.19
4		40.452	2412.25	0.16
5		43.634	657.87	0.04
6		44.235	617.35	0.04
7		46.943	1643.26	0.11
8		47.108	6983.38	0.47
9		47.723	589.15	0.04
10		47.963	348174.25	23.54
11		48.215	194.73	0.01
12		49.113	1216.37	0.08
13		51.307	2610.64	0.18
14		53.637	69816.92	4.72
15		53.970	474530.92	32.08
16		54.009	235074.89	15.89
17		54.022	212778.01	14.38
18		54.286	425.99	0.03
19		54.697	103882.53	7.02
20		55.023	787.26	0.05
21		55.252	1024.68	0.07
22		56.590	183.85	0.01
23		57.811	1396.70	0.09
24		60.185	1010.55	0.07
25		60.876	4851.91	0.33
26		66.815	1154.17	0.08
27		70.722	961.47	0.06
28		71.408	416.40	0.03
29		74.566	260.90	0.02
30		74.596	205.87	0.01
			1479287.61	100.00

Missing Component Report
Component Expected Retention (Calibration File)

All components were found

Fig. 2. Retention time of Tasar silkworm pupal oil

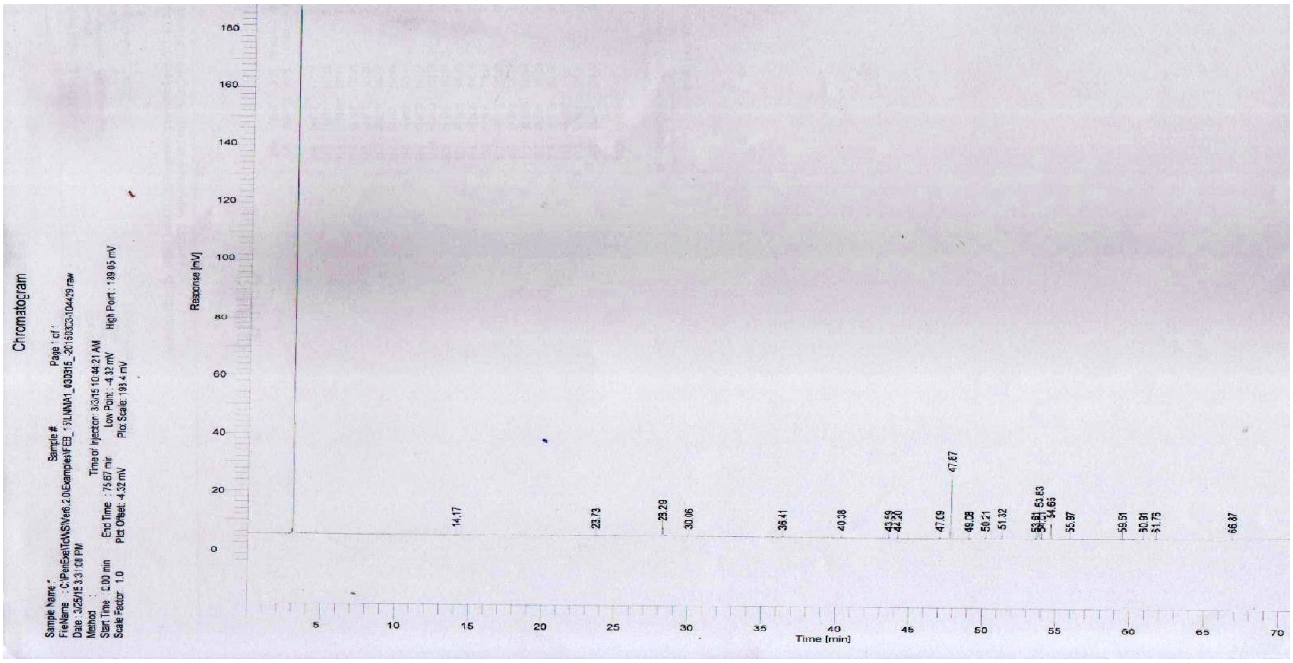

Fig. 3. GC-MS of Tasar silkworm pupal oil

first test tube used as control media with bacteria and in another four test tube put oil of Tasarsilk worm pupal oil at different concentration of 12.5, 25, 50, 100 µl/ml respectively. The experiment was done in triplicate. All the test tubes were incubated at 37^{0}C for 72 hours. After incubation, OD (optical density) was taken at 610 nm by spectrophotometer.

The MIC test allowedus to determine the lower concentration of Tasar silkworm pupal oil, which inhibited the growth *Staphylococcus sciuri* completely. The percent growth inhibition of the bacteria in presence of different concentration of Tasar silkworm pupal oil was calculated by following method.

$$\text{Percentage growth inhibition} = \frac{\text{(1-OD of test) x 100}}{\text{OD of control}}$$

RESULTS AND DISCUSSION

Fourier Transform Infrared Spectroscopy (FTIR) analysis of Tasar silkworm pupa oil

FTIR analysis (Fig. 1 and Table 1) of Tasar silkworm pupal oil insinuated the presence of alkanes. The absorption arising from C-H str; m, s region stretching in alkanes occurs in region between 2960-2850 cm^{-1} which peak value are 2932.5 and 2853.5. C-H bend; m between 1485-1440 and 1470-1430 the peak are1463.2 for both. The C-H bend; w between 1485-1340 the peak 1463.2, 1417.9, 1377 and C-C str; w between the region 1300-800 and its peak are 1237.2, 1160.3, 1099.6 and 1068.6, 967.2, 915 and 866.6 are in Tasar silkworm pupal oil. The C=C-H stretching mode of unconjugated alkenes usually shows region 3100-3000 its peak value is 3010.8, C-H; m 3040-3010 showed peak 3010.8, C-H bend; s 970-960 its peak is 967.2 and C-H bend; s 915-905 showed 915.0 peak value in Tasar pupal oil. The alkynes and Cycloalkanes C-H str; m 3100-2920 it's peak are 2923.5 and 3010.8 in Tasar pupal oil. For aromatic compound Ar-H str; v 3050-3000 it's peak are 3010.8 and C-H bend; s 900-700 it's peak are 866.6, 792.3, 720.8. C-F str; s for halogen compound between 1400-1000 it's peak are 1377.0, 1237.2, 1160.3, 1099.6, 1068.6 and C-Cl str; s region 800-600 it's peak are 792.3 and 720.8. The alcohols showed O-H str; v, sh between 3700-3500 and 3570-3450 showed 3628.7 and 3467.5 peak while O-H str; s 3000-

Table 2. GC-MS analysis of Tasar silkworm pupae oil

Compound name	Molecular Formulae	Molecular Weight g/mol	Retention time	Peak value	Area (uV*sec)	Area (%)
Palmitic acid	$CH_3(CH_2)_{14}$ COOH	256.42	47.963	40.62	348174.25	23.54
Linoleic	$CH_3(CH_2)_4$ CH=CHCH$_2$CH= CH$(CH_2)_7$COOH	280.44	53.637	45.69.	69816.92	4.72
Linolenic acid	CH_3CH_2CH= CHCH$_2$CH= CH$(CH_2)_7$COOH	278.43	53.970	45.94	474530.92	32.08
Oleic acid	CH3(CH2)7CH=CH(CH2)7 COOH	282.46	54.009	46.04	235074.89	15.89
Stearic acid	$CH_3(CH_2)_{16}$ COOH	284.47	54.022	46.79	212778.01	14.38

Table 3. Minimum inhibitory concentration of Tasar silkworm pupal oil and Streptomycin against *Staphylococcus sciuri* strain CD97

Concentration (µl/ml)	Tasar pupal oil Mean±SD (OD) nm	% Growth inhibition by Tasar Pupal oil	Streptomycin Mean±SD (OD) nm	% Growth inhibition by Streptomycin
Control	0.878±0.05	-	0.325±0.01	-
12.5	0.862±0.06	15.71	0.139±0.00	264.26
25	0.783±0.006	24.62	0.0824±0.01	281.90
50	0.635±0.06	41.56	0.0158±0.00	302.34
100	0.564±0.01	49.62	0.0135±0.00	303.07
110	0.00±0.00	100	0.00±0.00	100

2500 showed 2923.5, 2853.5 peak value. In case of phenols C-O str; s between 1400-1310 and 1410-1300 showed peak1377.0 and1377.0. While in ether C-O str; s between 1270-1200, 1150-1070 showed peak 1237.0, 1099.6 and in 910 region no any peak. The aldehydes C-H str; w between 2900-2820 peak 2853.5. In case of ketones C=O str; s between 1725-1710, 1725-1700,1715-1690 are showed no peak while ~1745 show 1743.5 peak. The esters C=O str; s region 1750-1735 have peak 1743.5 while region 1730-1715 have no peak. The Lactones C=O str; s between 1760-1740 and 1750-1735 both region's peak are 1743.5. In saturated aliphatic acids C=O str; s between 1725-1700 and 1715-1694 both region's have no peak. The carboxylic acid O-H str; w, b between 3000-2500 it's peak are 2923.5, 2853.5. Acid anhydrides C=O str; s between 1790-1740 and 1770-1725 it's both peak are

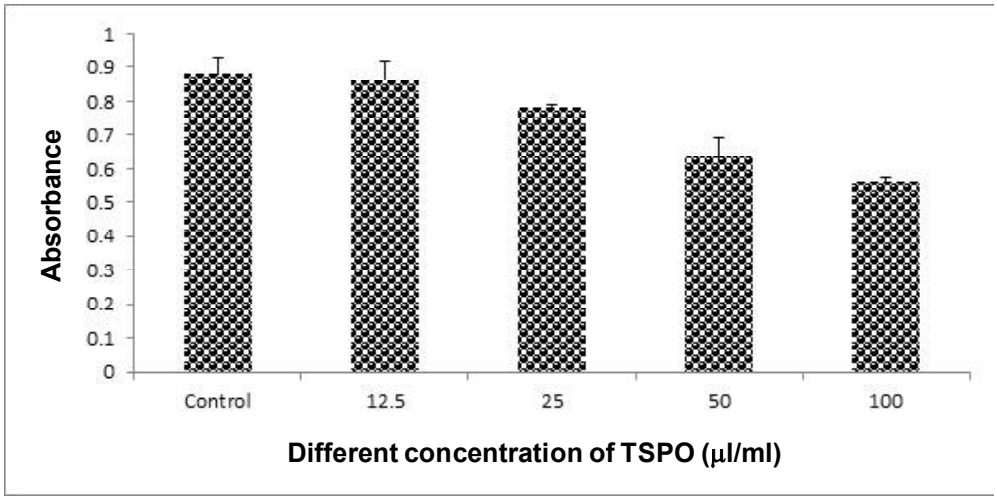

Fig. 4. Minimum inhibitory concentration of Tasar silkworm pupal oil (TSPO)

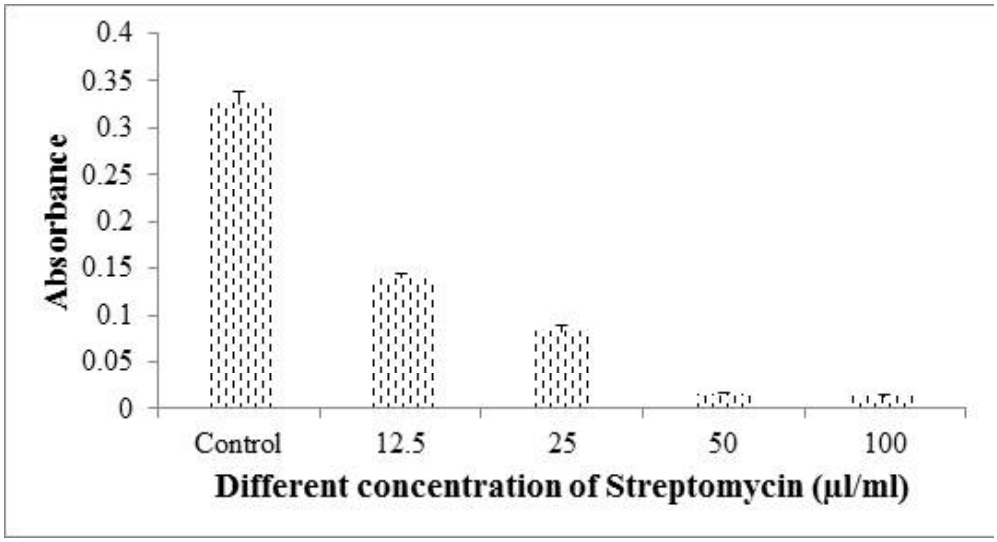

Fig. 5. Minimum inhibitory concentration of Streptomycin

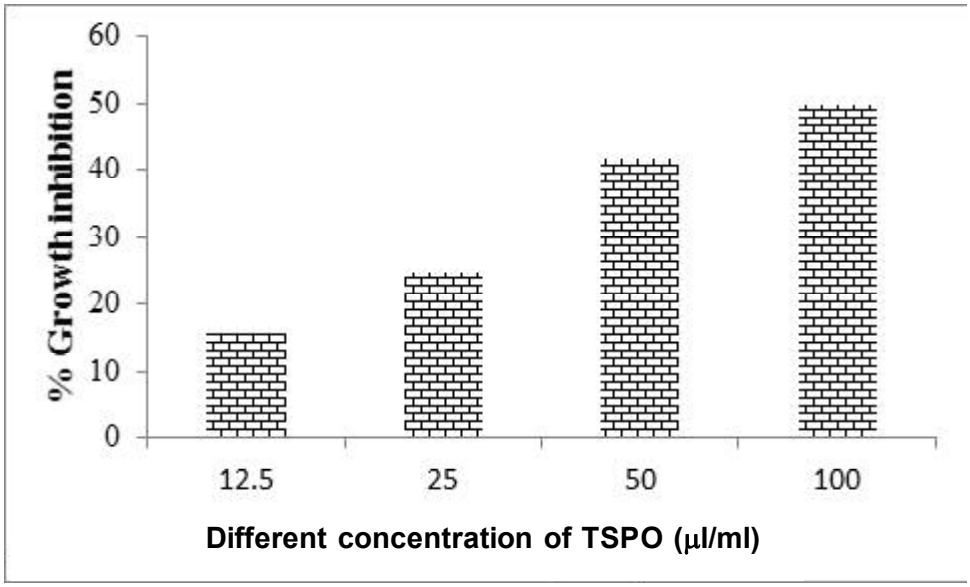

Fig. 6. Percentage of growth inhibition of *Staphylococcus sciuri*strain CD97 in presence of Tasar silkworm pupal il (TSPO)

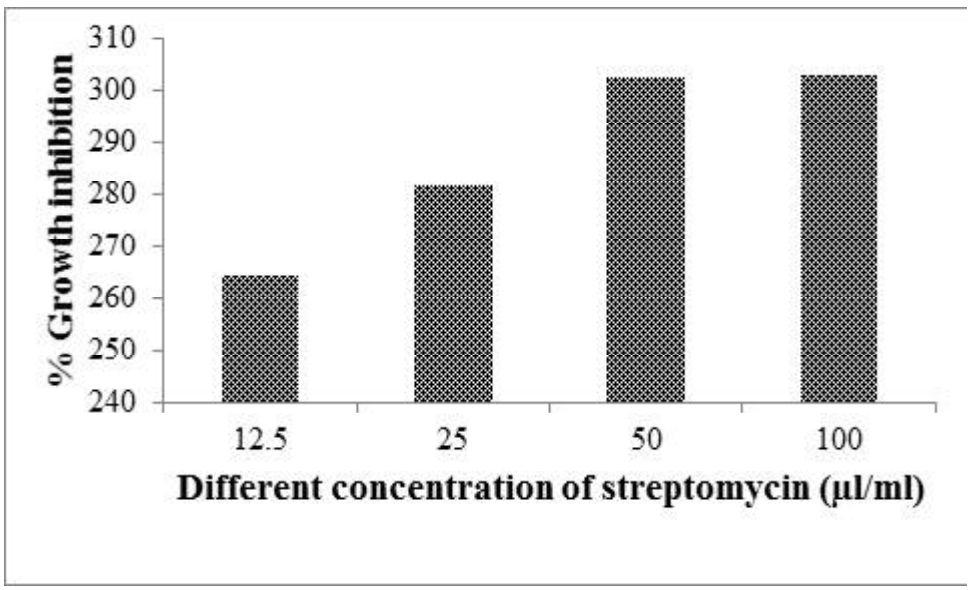

Fig. 7. Percentage of growth inhibition of *Staphylococcus sciuri*strain CD97 in presence of streptomycin

1743.5. The Lactams C=O str; s between 1760-1730 it's peak is 1743.5. While for amines N-H str; m region 3500-3300 showed 3467.5 peak in Tasar silkworm pupal oil. For nitro compound N=O str; s between 1375-1275 have no peak.

GC/MS Analysis of Fatty acid composition of Tasar silkworm pupal oil

The fatty acid composition of Tasar silkworm pupal oil were identified by gas chromatography mass spectroscopy technique and found the compounds namely palmitic acid, linoleic acid, linolenic acid, oleic acid and stearic acid whose retention time were 47.963, 53.637, 53.970, 54.009 and 54.022 respectively (Fig. 2; Table 2).

Minimum Inhibitory Concentration of Tasar silkworm pupal oil against *Staphylococcus sciuri* CD97

Tasar silkworm pupal oil was analyzed for its antagonistic potentiality against *Staphylococcus sciuri* strain CD97.The bacterial suspensions were prepared in Muller Hinton Broth (MHB) and growth were measured by spectrophotometer at 610 nm after 72 hours in terms of optical density.

The optical density (OD) of Tasar silkworm pupal oil and streptomycin decreases within creasing concentration of pupal oil and antibiotic (Table 3; Fig. 4,5,6,7); the percentage growth inhibition also increases by increasing the concentration of pupal oil and antibiotic (Table 3; Fig. 5,6). The optical density of Tasar pupal oil 0.564±0.011 at 100µl/ml was comparatively less than control 0.878±0.05. The value of optical density of streptomycin was 0.0135±0.00 at 100µl/ml in comparison to control 0.325±0.01 The decreasing optical density showed for minimum inhibitory concentration was 0.00±0.00 for both Tasar silkworm pupal oil and streptomycin at 110µl/ml.

Ramappa *et al.,* (2015) analyzed the chemical compounds of different Mulberry and Non Mulberry Silkworm Pupae Powder with FTIR and EDX. The present study completely focus on the FTIR and GC/MS analysis of *Antheraea mylitta* silkworm pupal oil and its antibacterial property against *Staphylococcus sciuri* strain CD97. The FTIR analysis insinuated the presence of alkanes, alkenes, alkynes, aromatic compounds, organic halogen compounds, alcohols, phenols, ethers, aldehydes, esters, carboxylic acid and amides.

Wen-Juan *et al.*(2012) studied the process optimization and composition determination of oak silkworm (*A. pernyi*) pupal oil by using supercritical carbon dioxide extraction method. In this method response, surface methodology was applied to optimize the parameters of SC-Co2 extraction, including extraction pressure, temperature, time and CO_2 flow rate on the yield of oak silkworm pupal oil. The finest extraction circumstance for oil yield inside the experimental series of the variables researched was at 28.03 MPa, 1.83 h, 35.31°C and 20.26 L/h as flow rate of CO_2. Under this condition, the oil yielded was about 26.18%. The oak silkworm pupal oil contains eight fatty acids, and is rich in unsaturated fatty acids and alpha-linolenic acid.

The GC/MS analysis of Tasar silkworm pupal oil showed the presence of palmitic acid, linoleic acid, linolenic acid, oleic acid and stearic acid. Similarly, Qian (1997) investigate the free fatty acid of silkworm pupal oil and found presence of compound namely were teradecyl acid, octadecanoic acid, hexadeene acid, hexadecanoic acid, linoleic acid, linolenic acid and oleinic acid whose content (%W/protein W) were 0.380,7.110, 0.490, 20.90, 7.470, 30.50 and 33.10 respectively.

Priyadarshini and Revanasiddaiah (2013) studied the fatty acid composition in pupal oil of *Philosamia ricini* (Eri silkworm pupae) by gas chromatography and found twelve fatty acids. The major fatty acids are linolenic acid, palmitic acid, oleic acid. The other fatty acid such as caproic acid, caprillic acid, capric acid and lauric acids were in the range of 0.01-0.07%. Some fatty acids found in trace amount are myristic acid, stearic acid, linoleic, bhenic acid and eruic acids. The unsaturated fatty acids dominated over the saturated fatty acids and beside this, the fatty acid pattern varied in both male and female pupae.

CONCLUSION

The present experiment was an attempt to study the spectral chemical composition of *A. mylitta* (Tasar) silkworm pupal oil using FTIR, GC-MS and to analyze antibacterial activity of *A. mylitta* pupal oil against *S. sciuri* strain CD97. The FTIR analysis of Tasar pupal oil proved the presence of alkanes, alkenes, alkynes, aromatic compounds, organic halogen compounds, alcohols, phenols, ethers, aldehydes, esters, carboxylic acid and amides while, GC-MS analysis revealed the presence of Palmitic acid, Linoleic acid, Linolenic acid and Stearic acid. The MIC test revealed that *A. mylitta* pupal oil significantly inhibited the growth of *S. sciuri* strain CD97 at 100 and 110 µl/ml concentrations.

ACKNOWLEDGEMENT

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Comparative Study of Antioxidant Potential of Mulberry and Non-Mulberry Silkworm Pupal Oil

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Silkworm pupae have a lot of antioxidant potential and can be used as a natural medicine to support human health. Winitchai (2011) extracted pupal oil from five native Thai silkworm varieties by using Soxhlet method showed free radical scavenging activity. In 2014, Deori et al., studied the antioxidant activity of pupae of the Muga and Eri silkworm and concluded that, the pupae could be used as natural antioxidants in food products.

Fish oil is a rich source of omega-3 polyunsaturated fatty acids, especially Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA). But, one of the major hurdles related with release of oils high in polyunsaturated fatty acid (PUFA), is the vulnerability of these oils to oxidative decline. Lipid oxidation is a serious problem throughout food processing and storage, as it decreases food quality and nutritive value. Therefore, antioxidants have been integrated to avoid oxidative instability of food components and to avert improvement of off-flavor compounds in food products (Yu et al., 2002). Many natural antioxidants with high antioxidant property have been incorporated in food products (Aligiannis et al., 2003;Athukorala et al., 2003).According to WHO (2003), It is suggested to ingestion n-3 fatty acids especially EPA and DHA, therefore efforts have been made to integrate marine oils, rich in n-3 PUFA, into diverse food products (Trautwein, 2001). The food products enriched within-3 PUFA is impeded by their high vulnerability towards oxidative decline.

The Polyunsaturated fatty acids (PUFAs) of silkworm pupa oil are considered as an excellent resource of á-linolenic acid (ALA). In pharmaceutical, the application of ALA is very essential. It generally dispersed in plant (Ihara et al., 2000) as well as in animal (Salazar et al., 2009) materials, which is extremely essential for human nutrition and disease prevention (Christensen et al., 2005). ALA has been used to prevent a variety of diseases such as cardiovascular (Roupas et al., 2006), hypertension, inflammatory and autoimmune disorders, (Ferrucci et al., 2006), depression and certain disrupted neurological functions (Christensen et al., 2005). As, it has been reported to generate Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) in the body, by a series of chain elongation and desaturation (Trattner et al., 2008). Therefore, ALA is a PUFA with important bioactivity and is used extensively in the medicine, food and cosmetic industries.

Silkworm pupae oil contains a mixture of essential fatty acids with bioactivity, therefore used as raw material for cosmetics. It is used for manufacturing of soaps and moisturizers (Kotake-Nara et al., 2002). Glutamic acid (18.3%), histidine (14.6%) and alanine (10.2%) are the most common amino acids present in silkworm pupae. Silkworm pupal oil is used as antioxidative to prevent aging. Tyrosinase inhibition activity is shown by None Ruesee silkworm pupal oil which is used to determine the inhibition of melanin formulation in the skin. In addition to that tyrosinase is a copper containing monooxyginase enzyme which can be found in fungi and higher plants and animals. It is known to be a key enzyme in melanin biosynthesis (Leibovitz et al., 2006, Gutierrez et al., 2006). To the best of our knowledge, there have been no comparative studies on the antioxidant potential of four different species of mulberry and non-mulberry silkworm pupal oil of i.e. *Antheraea mylitta*, *Samia ricini*, *Antheraea assamensis* and *Bombyx mori*. Therefore, the main objective of the present work was to extract oil from all four mulberry and non-mulberry silkworm species, and assess their relative antioxidant property by using DPPH assay method.

The Silkworm pupal oil has innumerable health benefits and this research article will try to throw light on the medicinal value of omega-3 which accounts for its health benefits. The therapeutic benefits of omega-3 fatty acids, which are abundant in certain fish oils, have long been known, dating back to at least the 1950s, when cod liver oil was found to be effective in treating ailments like eczema and arthritis.

MATERIALS AND METHODS

Collection of Test organisms:

The cocoons of three different silkworm varieties i.e. Eri, Tasar and Muga were collected from different places. The cocoons of Eri (*Samia ricini*) were taken from Banvasi Seva Ashram, Govindpur via Tura Sonbhadra, Uttar Pradesh. The cocoons of second species i.e. Tasar (*Antheraea mylitta*) was collected from Tasar silk Koya Market Dudhi, Pradesic Co-operative Sericulture Federation Limited Uttar Pradesh (Office of Assistant Director Sericulture Sonbhadra) while the cocoon of Muga (*Antheraea assamensis*) were obtained from Central Muga Eri Research and Training Institute, Central Silk Board, Ministry of Textiles, Govt. of India, Assam. The cocoon of fourth silkworm variety (*Bombyx mori*) were cultured at Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar (A Central) University, Lucknow, by conducting silkworm rearing as per procedure recommended by Krishnaswami et al., (1973). The solvent (Petroleum Ether 40-60°C) and Quality filter papers 11cm (110R) G-1necessary for DPPH assay method were purchased from Bionic Enterprises, Lucknow, Uttar Pradesh, India.

Extraction of Silkworm Pupal Oil

The mulberry and non-mulberry silkworm pupae were

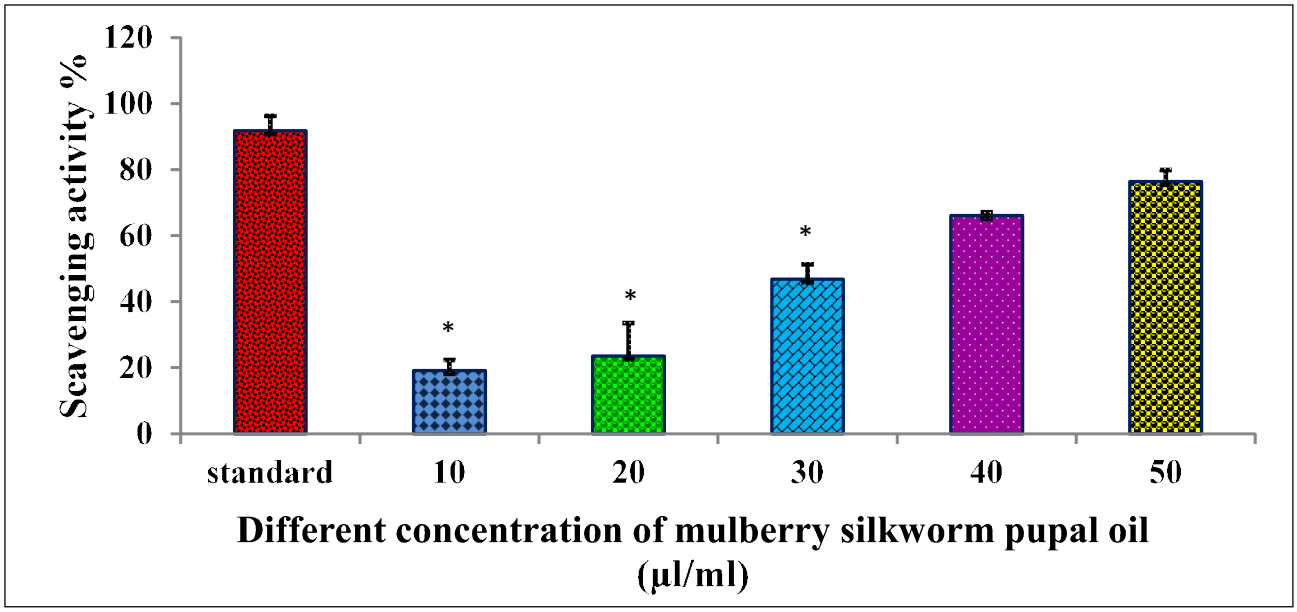

Fig. 1. Bar graph with standard deviation represents the antioxidant potentiality of different concentrations i.e. 10, 20, 30, 40 and 50µl/ml of Mulberry silkworm pupal oil by DPPH assay method. Mean separation at each concentration is significant by LSD at 5% level.

ground by using a mixer grinder to tear open the chitinous coating and to take out pupal matter. The extraction of oil from the pupal powder was carried out by employing maceration method (Nipha and Arunyakorn 1997). For this method; we took 10g dry pupae powder of the silkworm in a 100 ml of reagent bottle and added about 30ml of solvent petroleum ether up to a level completely submerging the powder. The reagent bottle was closed tightly, and sealed with glycerin to avoid evaporation. The reagent bottle was kept at room temperature for the period of seven days or more until the colour of the solvent turned yellow. The contents of regent bottle were filtered into petridishs and the filtrates were kept open in shed to evaporate the volatile solvent in which the oil was extracted.

Antioxidant Activity Assay

Determination of free radical scavenging activity using DPPH method

The free radical scavenging activity of mulberry oil were measured by the spectrophotometric method for the assay of hydrogen donating of DPPH by following procedure described by Sanja et al., (2009). Different concentration of oil samples as 10 µl/ml, 20 µl/ml, 30 µl/ml, 40 µl/ml and 50 µl/ml and ascorbic acid (0.1 mg/ml) as standard

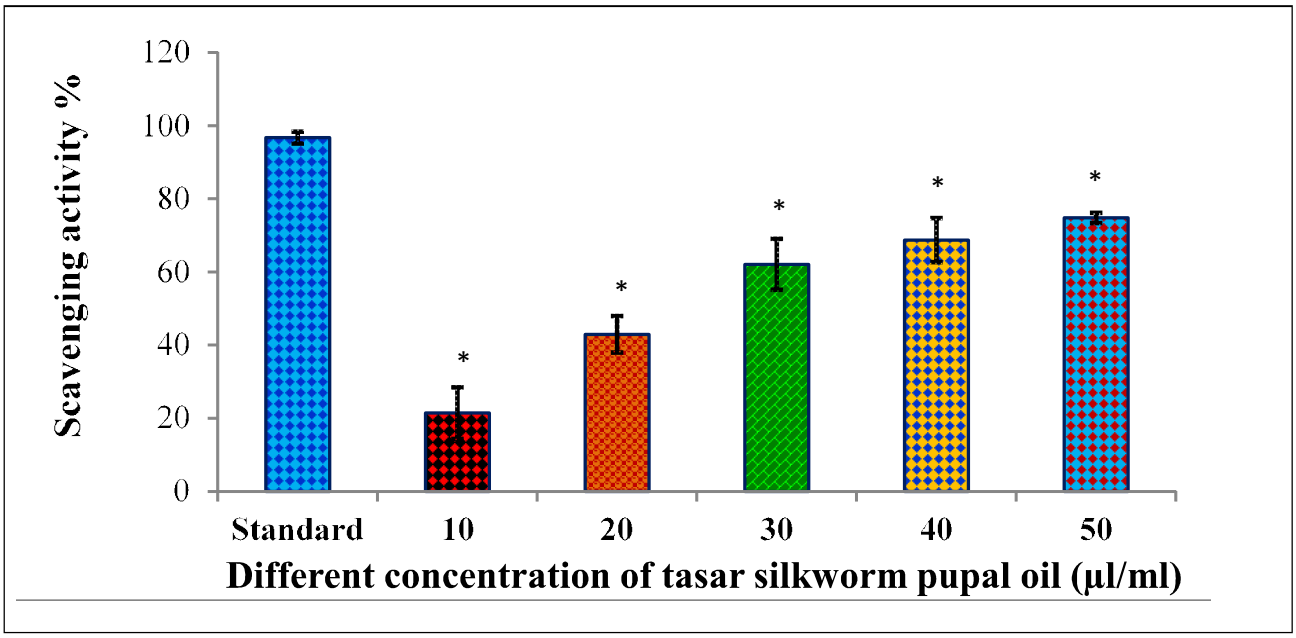

Fig. 2. Bar graph with standard deviation represents the antioxidant potentiality of different concentrations i.e. 10, 20, 30, 40 and 50µl/ml of Tasar silkworm pupal oil by DPPH assay method. Mean separation at each concentration is significant by LSD at 5% level.

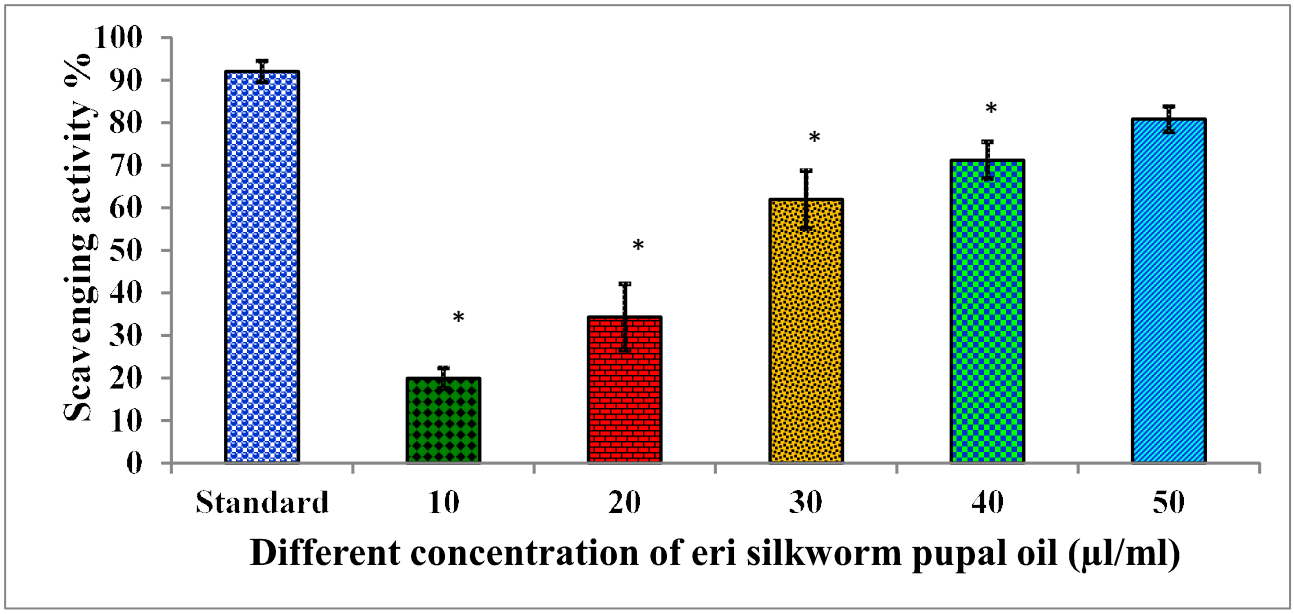

Fig.3. Bar graph with standard deviation represents the antioxidant potentiality of different concentrations i.e. 10, 20, 30, 40 and 50µl/ml of Eri silkworm pupal oil by DPPH assay method. Mean separation at each concentration is significant by LSD at 5% level.

were prepared in methanol solvents separately. Thereafter 150 µl of 0.004% DPPH reagent was added and final volume makeup 3 ml with the help of methanol solvent. The reaction mixture was mixed thoroughly and left for incubation at 15 min at room temperature in dark condition. The absorbance was measured at 516 nm using a spectrophotometer (Model No. Laptonic NT 2910) and antioxidant activity was expressed as percentage inhibition. The percent reductions were calculated by using the following equation and IC50 was calculated by using by using IBM SPSS (Version 20) package.

$$\text{FRSA (\% Antiradical activity)} = \left[\frac{A_0 - A}{A_0}\right] \times 100$$

Where,

FRSA =Percentage of Free Radical Scavenging Activity,

A_0=Absorbance of DPPH in the absence of the sample

A =Absorbance of DPPH in the presence of sample

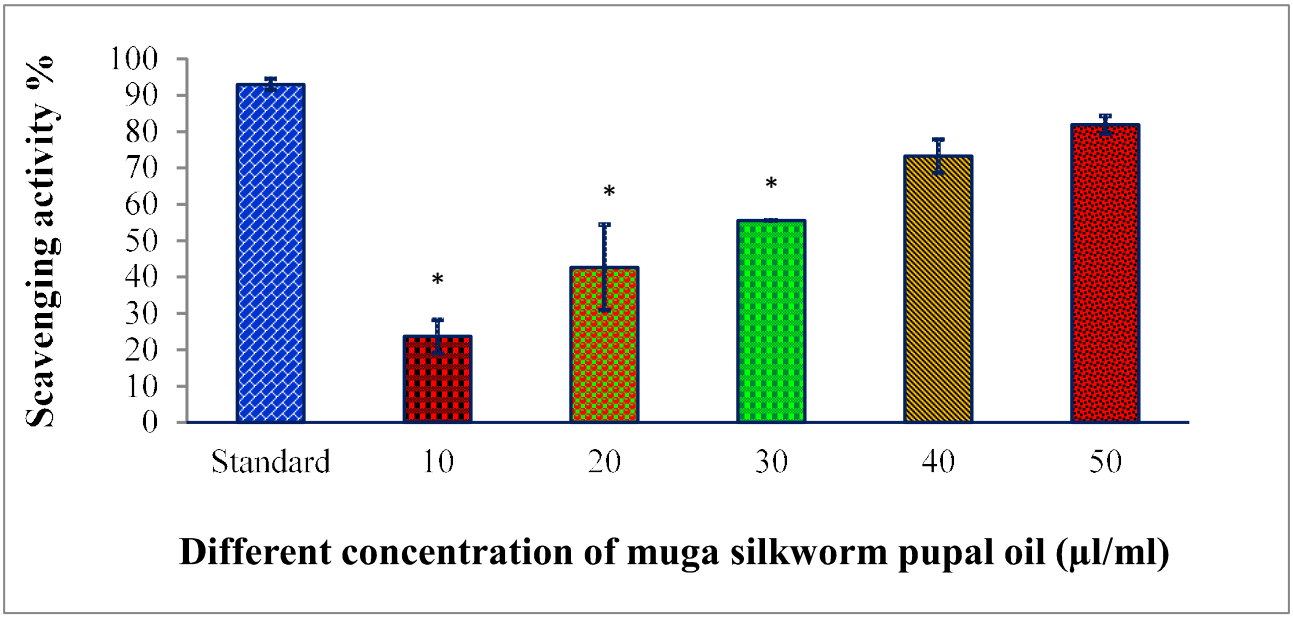

Fig. 4. Bar graph with standard deviation represents the antioxidant potentiality of different concentrations i.e. 10, 20, 30, 40 and 50µl/ml of Muga silkworm pupal oil by DPPH assay method. Mean separation at each concentration is significant by LSD at 5% level.

Statistical Analysis

The assay was carried out in triplicate. Data were analyzed by applying one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test and results were analyzed as mean ± standard deviation (SD). Levels of significance were tested at the level of pÂ0.05by using IBM SPSS (Version 20) package.

RESULTS AND DISCUSSION

DPPH is a stable free radical that accepts hydrogen radical to become a stable diamagnetic molecule. The reduction capacity of DPPH radical is determined by the decrease in its absorbance at 516 nm induced by antioxidants. The maximum absorption maximum of stable DPPH radical in ethanol was at 516 nm. The antioxidant decreases the absorbance of DPPH radicals, as the reaction between antioxidant molecules and radical progresses, results in the scavenging of the radical by hydrogen donation. Fig. 1, 2, 3 and 4 illustrates increase scavenging of DPPH radical in dose dependent manner due to the scavenging ability of the mulberry pupal oils.

Deori et al., (2014) investigated the antioxidant activity of pupae of the Eri (*Samia ricini*) and Muga silkworm's (*Antheraea assamensis*) by preparing methanolic pupae extract (MPE). In this study, antioxidant activity was determined by using 1-1 diphenyl 2 picryl hydrazyl (DPPH) radical, and reducing power assay method. The methanolic pupae extract (MPE) Eri and Muga showed good DPPH radical scavenging activity with IC_{50} value of 18.71ìg/mL and 25.83ìg/mL respectively. The MPE of muga pupae had phenolic (12.2 mg catechin/g) and flavanoid content (5.45 mg quercetin/g) and MPE of Eri pupae had significant ($P < 0.05$) higher phenolic (17.69 mg catechin/g) and flavanoid (3.47 mg quercetin/g) content. Therefore, pupae could be used as natural antioxidants in food products. However, the antioxidant activity of Mulberry silkworm pupal oil was lower than that of standards. Winitchai et al., (2011) extracted oil from None Ruesee (silkworm pupae) by the maceration method gave the highest free radical scavenging activity. Moreover, oil extracted by the Soxhlet method from None Ruesee gave the highest tyrosinase inhibition activity, but was lower than that of standards of vitamin C and kojic acid. The study indicated that oil from native Thai silkworm pupae could be used as an alternative in the food and cosmetic industries. In this study, presence of linolenic acid (ALA, 18:3 n-3) in mulberry and non-mulberry silkworm pupal oil showed antioxidant activity. The antioxidant activity of the oil might be due to the presence of linolenic acid (ALA, 18:3 n-3) and its metabolic yield, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Kaithwas and Majumdar (2012) studied *in-vitro* antioxidant activity of *Linum Usitatissimum* fixed oil by using DPPH assay. DPPH is a stable free radical and is reduced by accepting an electron or hydrogen radical to become stable diamagnetic hydrazine molecule (Soares et al., 1997). Suggesting potential antioxidant property of the mulberry, tasar, eri and muga silkworm pupal oil were same. Deori and Devi (2011) found the IC_{50} value of aqueous sericin extract of muga silkworm which was 8µl/ml. The lower IC_{50} value indicates a higher free radical scavenging activity.

CONCLUSION

The pupal oil is an exciting subproduct obtained after the extraction procedure of silk threads. The DPPH assay of the Mulberry silkworm pupae oil showed some scavenging activity, but it was lower than that of standard vitamin C. The silkworm pupae oils contain phospholipids and tocopherol, which forceally act as antioxidants. The study showed the opportunity of selecting proper varieties of silkworm pupae as sources of protein and fat that could be developed for food and cosmetic products. Thus, it is possible to infer that mulberry and non-mulberry silkworm oil could be used to balance human nutrition requirements, as a complementary food, as an alternative antioxidant.

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Assessment Antioxidant Properties of Mulberry, Tasar, Eri and Muga Silkworm Pupal Oil by Superoxide Assay

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Natural antioxidant present in the silkworm pupae scavenges damaging free radical from our body. Free radical is any species which is able to independent existence that contains one or more unpaired electrons which reacts with other molecule by captivating or philanthropic electrons and implicated in various pathological conditions (Madhavi et al., 1996). Silkworm pupae have vitamin B_2, which can be important to avoid the serious effects of vitamin B_2 deficiency (Kwon et al., 2012). The fermented silkworm powder has showed defensive effect in alcohol-induced hepatotoxicity in a rat model (Cha et al., 2012). Silkworm pupae restrain 45–55 % protein content on a dry matter basis, which can considerably enhance the hemoglobin and serum total protein in rats, producing protective effects on the liver in carbon tetrachloride-induced rat hepatic injury (Shi et al., 1990, Yang et al., 2002). Meetali et al., (2014) also evaluated the antioxidant and antigenotoxic property of muga silkworm pupa, so, now a days it is recommended that silkworm pupae can be utilize as natural antioxidants in various food products.

Silkworm pupal oil of five native Thai silkworm varieties was showed free radical scavenging activity Winitchai (2011). The antioxidant activity of pupae of the Muga and Eri silkworm and concluded that, the pupae could be used as natural antioxidants in food products Deori et al., (2014).

The antioxidants have been integrated to avoid oxidative instability of food components and to avert improvement of off-flavor compounds in food products (Yu et al., 2002). Many natural antioxidants with high antioxidant property have been incorporated in food products (Aligiannis et al., 2003; Athukorala et al., 2003). According to WHO (2003), It is suggested to ingestion n-3 fatty acids especially EPA and DHA, therefore efforts have been made to integrate marine oils, rich in n-3 PUFA, into diverse food products (Trautwein, 2001). The food products enriched within-3 PUFA is impeded by their high vulnerability towards Omega oxidative decline. (Change the language).

The Polyunsaturated fatty acids (PUFAs) of silkworm pupa oil are considered as an excellent resource of á-linolenic acid (ALA). In pharmaceutical, the application of ALA is very essential. It generally dispersed in plant (Ihara et al., 2000) as well as in animal (Salazar et al., 2009) materials, which is extremely essential for human nutrition and disease prevention (Christensen et al., 2005). ALA has been used to prevent a variety of diseases such as cardiovascular (Roupas et al., 2006), hypertension, inflammatory and autoimmune disorders, (Ferrucci et al., 2006), depression and certain disrupted neurological functions (Christensen et al., 2005). As, it has been reported to generate Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) in the body, by a series of chain elongation and desaturation (Trattner et al., 2008). Therefore, ALA is a PUFA with important bioactivity and is used extensively in the medicine, food and cosmetic industries. (Change the language).

Silkworm pupae oil contains a mixture of essential fatty acids with bioactivity, therefore used as raw material for cosmetics. It is used for manufacturing of soaps and moisturizers (Kotake-Nara et al., 2002). Glutamic acid (18.3%), histidine (14.6%) and alanine (10.2%) are the most common amino acids present in silkworm pupae. (Change the language).

Silkworm pupal oil is used as antioxidative to prevent aging. Tyrosinase inhibition activity is shown by None Ruesee silkworm pupal oil which is used to determine the inhibition of melanin formulation in the skin. While Tyrosinase is a copper containing monooxyginase enzyme which can be found in fungi and higher plants and animals. It is known to be a key enzyme in melanin biosynthesis (Leibovitz et al., 2006, Gutierrez et al., 2006). To the best of our knowledge, there have been no comparative studies on the antioxidant potential of four different species of mulberry and non-mulberry silkworm pupal oil of i.e. *Antheraea mylitta*, *Samia ricini*, *Antheraea assamensis* and *Bombyx mori*. Therefore, the main objective of the present work was to extract oil from all four mulberry and non-mulberry silkworm species, and assess their relative antioxidant property by using superoxide assay method.

MATERIALS AND METHODS

Collection of Test organisms

The cocoons of three different silkworm varieties i.e. Eri, Tasar and Muga were collected from different places. The cocoons of Eri (*Samia ricini*) were taken from Banvasi Seva Ashram, Govindpur via Tura Sonbhadra, Uttar Pradesh. The cocoons of second species i.e. Tasar (*Antheraea mylitta*) was collected from Tasar silk Koya Market Dudhi, Pradesic Co-operative Sericulture Federation Limited Uttar Pradesh (Office of Assistant Director Sericulture Sonbhadra) while the cocoon of Muga (*Antheraea assamensis*) were obtained from Central Muga Eri Research and Training Institute, Central Silk Board, Ministry of Textiles, Govt. of India, Assam. The cocoon of fourth silkworm variety (*Bombyx mori*) were cultured at Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar (A Central) University, Lucknow, by conducting silkworm rearing as per procedure recommended by Krishnaswami et al., (1973). The solvent (Petroleum Ether 40-60°C) and Quality filter papers 11cm (110R) G-1necessary for DPPH assay method were purchased from Bionic

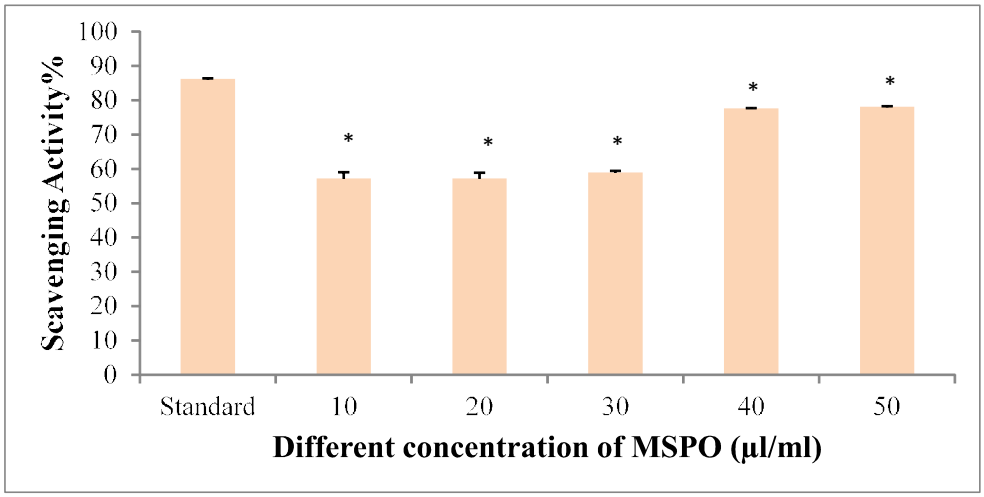

Fig. 1. Bar graph with standard deviation represents the antioxidant potentiality of different concentrations i.e. 10, 20, 30, 40 and 50µl/ml of (MSPO) Mulberry silkworm pupal oil by superoxide assay method. Mean separation at each concentration is significant by LSD at 5% level.

Enterprises, Lucknow, Uttar Pradesh, India.

Extraction of Silkworm Pupal Oil

The mulberry and non-mulberry silkworm pupae were ground by using a mixer grinder to tear open the chitinous coating and to take out pupal matter. The extraction of oil from the pupal powder was carried out by employing maceration method (Nipha and Arunyakorn 1997). For this method; we took 100g dry pupae powder of the silkworm in a 1000 ml of reagent bottle and added about 300ml of solvent petroleum ether up to a level completely submerging the powder. The reagent bottle was closed tightly, and sealed with glycerin to avoid evaporation. The reagent bottle was kept at room temperature for the period of seven days or more until the colour of the solvent turned yellow. The contents of regent bottle were filtered into petridishs and the filtrates were kept open in shed to evaporate the volatile solvent in which the oil was extracted.

Super oxide scavenging activity of mulberry and non-mulberry silkworm pupal oils by using alkaline DMSO method:

Chemicals and Reagents

Dimethyl sulfoxide was purchased from Biotech park, Nitro-blue Tetrazolium was purchased from CDRI Lucknow.

Preparation of standard solution

10 mg of ascorbic acid dissolved in 10 ml of absolute alcohol. Dilutions of this solution with absolute alcohol were prepared to give the concentration of 10 ìl, 20 ìl, 30 ìl, 40 ìl and 50 ìl / ml.

Preparation of test sample

Dissolve 25 ml of mulberry and non-mulberry silkworm pupal oil in 25ml of dimethyl sulfoxide to give stock solution of 1 ml/ml. Dilution were done with same dimethyl sulfoxide to give concentrations of 10 µl, 20 µl, 30 µl, 40 µl, 50 µl/ml.

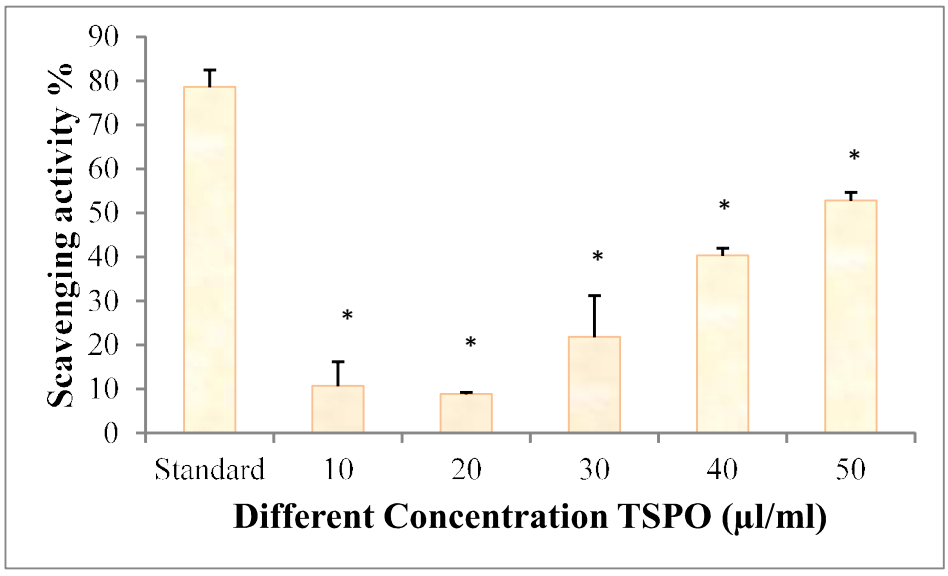

Fig. 2. Bar graph with standard deviation represents the antioxidant potentiality of different concentrations i.e. 10, 20, 30, 40 and 50µl/ml of (TSPO) Tasar silkworm pupal oil by superoxide assay method. Mean separation at each concentration is significant by LSD at 5% level.

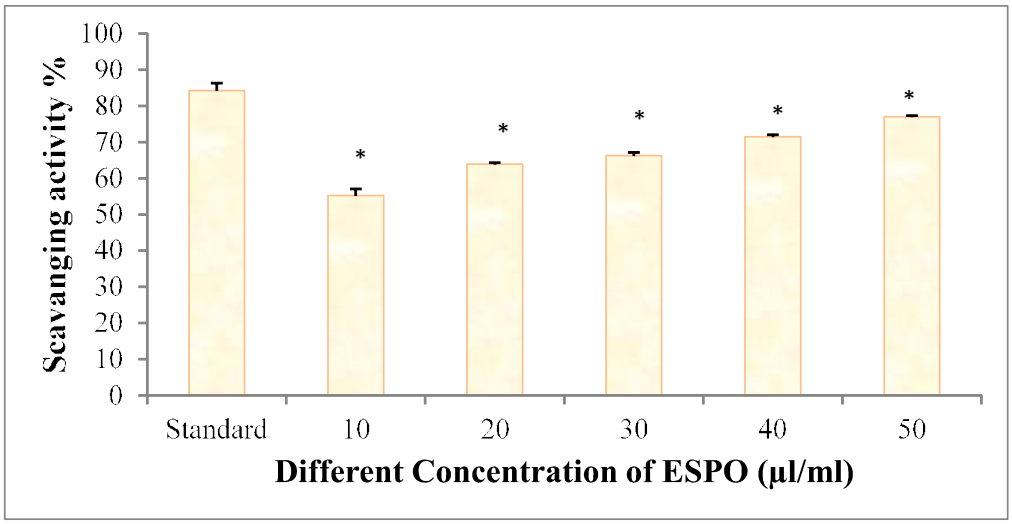

Fig.3. Bar graph with standard deviation represents the antioxidant potentiality of different concentrations i.e. 10, 20, 30, 40 and 50µl/ml of (ESPO) Eri silkworm pupal oil by superoxide assay method. Mean separation at each concentration is significant by LSD at 5% level.

Preparation of reagents

Alkaline DMSO: 1 ml alkaline DMSO containing, 5 mM NaOH in 0.1 mL absolute alcohol and 0.9 ml Dimethyl sulfoxide. NBT: 25 mg of nitro-blue Tetrazolium was dissolved in 25 ml of Dimehtyl sulfoxide to give concentration of 1 mg/ml.

Protocol for estimation of superoxide scavenging activity

To the reaction mixture containing 0.1 mL of NBT (1 mg/mL solution in DMSO) and 0.3 mL of the pupal oil and standard in DMSO, 1 mL of alkaline DMSO (1 mL DMSO containing, 5 mM NaOH in 0.1 mL alcohol) was added to give a final volume of 1.4 mL and the absorbance was measured at 560 nm. Pupal oil (100-600 il/ml) was added to a hydrogen peroxide solution (0.6ml, 40mM). 300 il of plain DMSO, 0.1 ml NBT solution and 1 ml alkaline DMSO was mixed and absorbance was taken at 560 nm and this was taken as control reading. The percentage of super oxide radical scavenging by the mulberry and non-mulberry silkworm pupal oil and standard compounds were calculated as follows:

$$\% \text{ Superoxide scavenging activity} = \frac{\text{test absorbance} - \text{control absorbance} \times 100}{\text{Test absorbance}}$$

Statistical Analysis

The experiment was carried in triplicate and the results are expressed as Mean±SD. The data were subjected to One-way analysis of variance (ANOVA). The difference of samples from control was determined by Least Significant Difference (LSD) test. P-Value of <0.05 was regarded as significant.

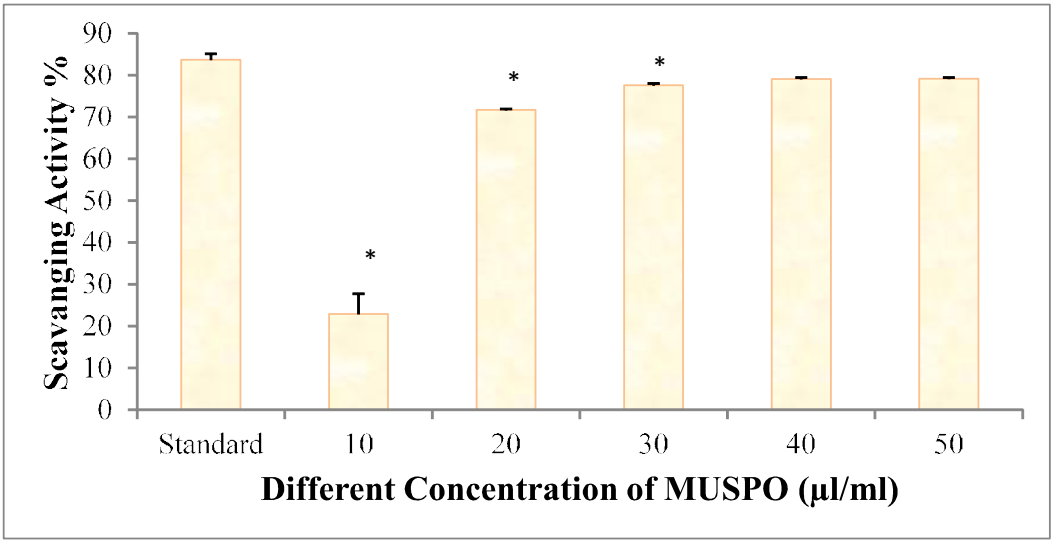

Fig. 4. Bar graph with standard deviation represents the antioxidant potentiality of different concentrations i.e. 10, 20, 30, 40 and 50µl/ml of (MUSPO) Muga silkworm pupal oil by superoxide assay method. Mean separation at each concentration is significant by LSD at 5% level.

RESULTS AND DISCUSSION

Super oxide scavenging activity pupal oil by alkaline DMSO method

Superoxide radicals are recognized as very injurious to the cellular component. Super oxide free radical was formed by alkaline DMSO which reacts with NBT to generate coloured diformazan. The mulberry, tasar, eri and muga silkworm pupal oil scavenges super oxide radical and thus inhibits formazan formation. Fig. 1, 2, 3 and 4 illustrates increase scavenging of superoxide radicals in dose dependent manner due to the scavenging ability of the mulberry and non-mulberry silkworm pupal oil.

Wiwat et al., (2015) lipids extracted from the adipose tissues of some catfish, as the Mekong giant catfish (*Pangasianodon gigas*), striped catfish (*Pangasianodon hypophthalmus*) and hybrid catfish (*Pangasius larnaudii x Pangasianodon hypophthalmus*) were carried out. Their saturated fatty acid and unsaturated fatty acid contents were not much different, but their polyunsaturated fatty acid contents were obviously different. Their n-3 fatty acid contents were 15.58, 0.83 and 4.36 g per 100 g, respectively, and their eicosapentaenoic acid (EPA):docosahexaenoic acid (DHA) contents were 3.23:4.24, 0.07:0.13 and 0.65:2.72 g per 100 g, respectively. Their antioxidant activities by ABTS assay were 3.21, 4.53 and 6.00 mM Trolox per 1 g, respectively. The same results was shown by mulberry, tasar, eri and muga silkworm pupal oil because it also have compound alpha linolenic acid which convert into EPA and DHA during metabolic process.

Ageing is nothing but it is a natural life process whose manifestations are familiar and unambiguous (Priya et al., (2015). The natural antioxidant mechanism of an organism may be insufficient and external dietary administration of antioxidant compounds play vital role in defense against ageing. The EPA and DHA act as natural antioxidant within our body.

Deori et al., (2014) investigated the antioxidant activity of pupae of the Eri (*Samia ricini*) and Muga silkworm's (*Antheraea assamensis*) by preparing methanolic pupae extract (MPE). The antioxidant activity was determined by using 1-1 diphenyl 2 picrylhydrazyl (DPPH) radical, and reducing power assay method. The pupae extract of Eri and Muga showed good DPPH radical scavenging activity with IC_{50} value of 18.71ig/mL and 25.83ig/mL respectively. The MPE of muga pupae had phenolic (12.2 mg catechin/g) and flavanoid content (5.45 mg quercetin/g) and MPE of Eri pupae had significant ($P < 0.05$) higher phenolic (17.69 mg catechin/g) and flavanoid (3.47 mg quercetin/g) content. Therefore, pupae could be used as natural antioxidants in food products. However, the antioxidant activity of Mulberry and non-mulberry silkworm pupal oil was lower than that of standards.

Winitchai et al., (2011) extracted oil from None Ruesee (silkworm pupae) by the maceration method gave the highest free radical scavenging activity. The study indicated that oil from native Thai silkworm pupae could be used as an alternative in the food and cosmetic industries. In the present study, presence of linolenic acid (ALA, 18:3 n-3) in mulberry, tasar, eri and muga silkworm pupal oil showed antioxidant activity due to the presence of linolenic acid (ALA, 18:3 n-3) and its metabolic yield, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3).

Kaithwas and Majumdar (2012) studied *in-vitro* antioxidant activity of *Linum Usitatissimum* fixed oil by using DPPH assay. DPPH is a stable free radical and is reduced by accepting an electron or hydrogen radical to become stable diamagnetic hydrazine molecule (Soares et al., 1997). Suggesting potential antioxidant property of the mulberry, tasar, eri and muga silkworm pupal oil were same. Deori and Devi (2011) found the IC_{50} value of aqueous sericin extract of muga silkworm which was 8µl/ml. The lower IC_{50} value indicates a higher free radical scavenging activity.

CONCLUSION

The pupal oil is an exciting subproduct obtained after the extraction procedure of silk threads. The superoxide assay of mulberry, tasar, eri and muga silkworm pupae oil showed some scavenging activity, but it was lower than that of standard vitamin C. The pupae oils contain phospholipids and tocopherol, which forceally act as antioxidants. The study showed the opportunity of selecting proper varieties of silkworm pupae as sources of fat that could be developed for food and cosmetic products. Thus, it is possible to infer that mulberry and non-mulberry silkworm oil could be used to balance human nutrition requirements, as a complementary food, as an alternative antioxidant.

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Chromium Accumulation by Eisenia Foetida in Modified Vermicompost Supplemented with Tannery Sludge

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Abstract: *The current study is aimed to analyze the potential of accumulation ofchromium present in tannery sludge by earth worms (Eisenia foetida). Chromium salt is commonly used for tanning of leather and it is present in trivalent and hexavalent form.As earthworms are known to clean up the soil from various pollutants and can accumulate metals from it. Therefore the earthworm provides an easy biological material to explore the potential of accumulation of chromium and limiting its presence in soil. Tannery sludge obtained from Unnao region of Uttar Pradesh was mixed with vermicompost in various concentrations and earthworms were exposed up to 50 days with same mixtures and finally estimated the rate of heavy metal (Cr) accumulation of earth worms' body tissuesusing AAS. The experimental results revealed that earthworms have accumulated 0.131 µg/l at 10% tannery sludge supplement where as 50 % it was 4.919 µg/l.*

1. INTRODUCTION

Environmental pollution from heavy metal contamination has increased to the point that it endangers human life in some areas and the reduction and eventual elimination of pollution in these areas is urgently needed. These unforeseen activities are increasing every passing day due to anthropological activities and urbanization. This continuous interference of human in environment is causing highest degree of pollution including metal toxicity. Metal toxicity especially chromium is one of the major elements causing severe effect on flora and fauna. Metal pollution disturbs soil ecosystem by affecting the structure of soil invertebrate population. Heavy metal toxicity and the danger of its accumulation in the food chain represent one of the major environmental and health problems of our modern society. Toxic metals are important category of pollutants and as such have major detrimental impacts on human health [6].

Earthworms can be exposed by direct dermal contact with heavy metal in soil solution or by ingestion of pore water polluted, food and /or soil particles [10]. In addition, earthworms are able to clean up the soil from various pollutants, and are able to accumulate heavy metal on their bodies from the soil too. The process of vermicomposting is used to treat heavy metal contaminated soil through bioaccumulation and conversion to non-toxic forms.[8] Earthworms have been widely used in the breakdown of a wide range of organic residues including sewage sludge, animal waste, crop residues and industrial refuse in producing vermicompost[5][9]. However the potential of remediation from tannery sludge is yet to be explored.

Vermiremedition is low cost, convenient, technology for combating soil and land pollution. Earthworms in general are tolerant to many chemical, contaminants in soil including heavy metal and organic pollutants and have been reported to bio accumulate some of them in their tissues. Vermicasting of earthworms contain enzyme like amylase, lipase, cellulase and chitinase, which continue to breakdown organic matter in the soil to release the nutrients and make it available to the plant roots even after they have been excreted[11][4].

Earthworms can bio-accumulate high concentrations of metals including heavy metals in their tissue without affecting their physiology [7]. Earthworms ingest metal with soil, change their ionic state in their gut through physiological action and render them in bio-accumulation form for plants when excreted out. Vermicomposting represents an excellent treatment method for contaminated soils, not only for waste reduction but also to recondition the soil. Vermicomposting constitutes a special form of composting in which earthworms metabolize and excrete a mixture of soil and organic matter. In the digestive system of these worms, microorganisms transform organic species (proteins, nucleic acids, fats, carbohydrates, etc.) into more stable products in the process of vermicompost[2].

2. MATERIALS AND METHODS

Earthworms were collected from Biotech Park, Lucknow, Uttar Pradesh for experimental purpose. The test was performed by using a mixture of tannery sludge with

vermicompost in different concentration i.e. 10%, 20%, 30%, 40%, 50% along with control. The different amendments of tannery sludge used were are 10%= 2700 gm vermicompost+300gm tannery sludge, 20%=2400 gm vermicompost+600gm tannery sludge, 30%=2100 gm vermicompost+900gm tannery sludge,40%=1800 gm vermicompost+1200gm tannery sludge, 50%=1500 gm vermicompost+1500gm tannery sludge and finally Control= 3000 gm vermicompost (without tannery sludge). Prior to this supplementation the amended Chromium content in tannery sludge was analyzed (Table-1).

These mixtures of tannery sludge & vermicompost were kept in clean plastic tubs. Each tub mixed with 3 kg of vermicompost and tannery sludge. Three replications from each concentration and control were used. Ten no. of earthworms (*Eisenia foetida)* weighing approximately 0.16-27gm and length 6cm of each were kept in the all experimental tubs. The moisture content of the mixture was maintained at 70-80 percent throughout the experimental period and plastic tubs were kept in the experimental room at 30-37^{o}C. After 50 days earthworms were collected from all three replicates of each concentration, further they were cleaned, dissected and collected 1gm of body tissue. Further, the body tissues were kept back into 100ml of conical flask. Added 15 ml of digestion mixture in 6:1 (HNO_3:$HClO_4$) (Nitric acid and Perchloric Acid) and heated on the sand bath. Heating was continued till brown fumes convert into white fumes and sample solution remains 0.5-1.0 ml at the bottom of flask. The volume of sample was made to 5ml by adding 1% nitric acid and filtered through Whatmann filter paper and analyzed with the help of Atomic Absorption Spectrophotometer (AA 240FS, Varion) at Department of Environmental Science, BBAU, Lucknow. The data obtained was analyzed by One Way analysis of Variance using SPSS software.

3. RESULTS

The experimental results of heavy metal (Chromium) content accumulated in earthworms after 50 days are summarized in table 2.

It was observed that the bioaccumulation of Chromium in earthworm body tissue varies as per the concentrations. It was found highest in 50% (2.829 µg/l) whereas lowest percentage of bioaccumulation was observed in 20% of tannery sludge (0.148 µg/l) followed by 10% (0.164µg/l), 30% (0.699) and 40% (1.354 µg/l).Earthworms survived well in pure vermicompost (control) as well as in experimental. Therefore, increasing trends of chromium absorption was observed with increased concentration of tannery sludge in vermicompost except 20%.

The statistical analyses revealed that variables show good inter relationship and its values are significant. Detail analysis is in Table-2.

One way analysis of Variance shows that there is a significant difference in the accumulation of Chromium of earthworm at different concentrations of tannery sludge ($F_{5,\ 12}$ = 5.113, P<0.05)

Table 1: Concentration of Chromium in tannery sludge mixture (µg/l)

S.N.	Concentration of tannery sludge in mixture	Chromium in tannery sludge
1	Control	0.285
2	10%	1.967
3	20%	1.989
4	30%	2.032
5	40%	3.255
6	50%	4.321

Table 2: Concentration of Chromium in earthworm body tissue (µg/l) at different concentration

S. No.	Tannery Sludge	R1	R2	R3	Mean
1	10%	0.131	0.150	0.210	0.164
2	20%	0.178	0.118	0.147	0.148
3	30%	0.451	0.912	0.734	0.699
4	40%	1.103	0.894	2.064	1.354
5	50%	1.211	2.358	4.919	2.829
6	Control	0.070	0.074	0.085	0.076

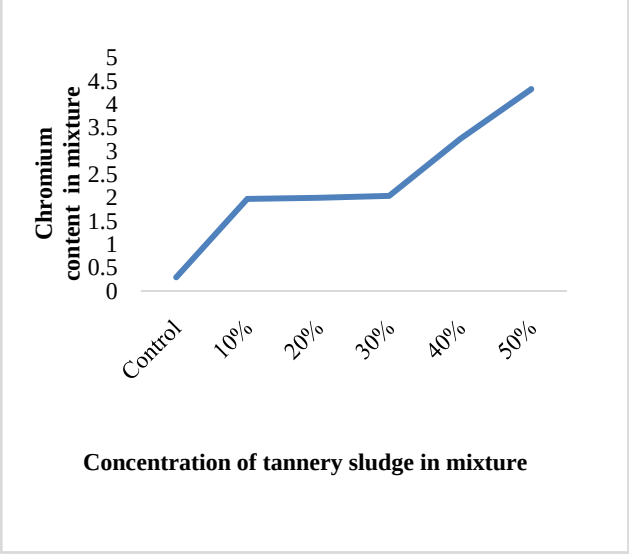

Fig. 1: Concentration of Chromium in tannery sludge mixture (µg/l)

4. DISCUSSION

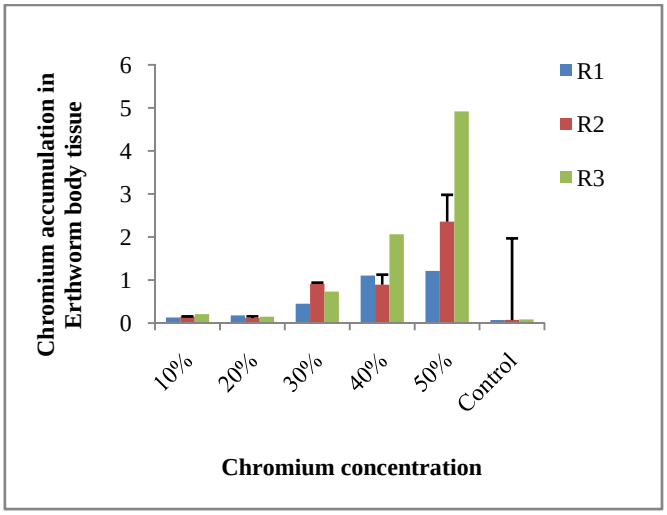

Fig. 2: Chromium accumulation in earthworm body tissue at different concentration

The accumulated Chromium in the body of earthworm shows its ability to digest Chromium. Current study also revealed that the uptake of Chromium by the earthworm is in increasing order as the concentration increases. However in one of the supplements (20%) of tannery sludge higher accumulation of Cr was not observed and its reasons are not known.

These findings were similar to that recorded by [13] [12] who indicated that earthworm activity increases the mobility and bioavailability of heavy metals in soil. Further a similar results obtained by[1] also substantiate the current results. The current study established the fact that earthworms (E*isenia fetida*) are competent enough to accumulate Cr content in their body tissue. Hence soil pollution can be eradicated by using earthworms. The study also reveals that heavy metal toxicity can cause serious damage to the ecosystem can be controlled by using biological organisms and earthworm can be used as a biomarker to access heavy metal pollution.

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Silkworm pupal oil: A novel source of Omega-3 fatty acids

The length of telomeres in chromosomes becomes reduced as the age grows. Omega-3-fatty acids which can not be synthesized by human body, however, can be obtained from fish oil, seafood, flax seed oil, soyabean oil and some other plant products etc., which may attenuate shortening of telomeres. Both the mulberry and non-mulberry silkworm pupae oil is a good source of Omega-3 fatty acids, which reduces the risk of cancer and cardiovascular diseases etc. Details.

Omega-3 fatty acids have become the buzz word in the present scenario as they have potential to act against cancer, cardiovascular diseases, inflammation, chronic disorders, mental health, cognitive aging, atopic diseases etc.

Omega-3 fatty acids are poly-unsaturated fats (PUFAs) which cannot be synthesized by the human body. Hence, it is inevitable to rely on external sources to get omega-3 fatty acids. Plant foods, fish oil, seafood, flaxseed oil and soybean oil are the major sources for omega-3 fatty acids. Thus, omega-3 fatty acid is a subject of intense research and development; and researchers have been trying to find out its useful linkages in human metabolism. One of the reports of an Australian researcher clearly indicates that increasing omega-3 poly-unsaturated fatty acid intake through supplementation may attenuate telomere shortening that occurs with age.

Further, studies have established that taking enough Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) can augment human health conditions, for which the fish and soya oils are good sources. Several studies also witnessed that people those who use a diet, rich in omega-3 fats are at lower risk of heart diseases than those who take less omega-3 in diet. Various omega-3 fatty acids namely, EPA, α-Linolenic acid (ALA), DHA, Eicosatrienoic acid (ETE), Eicosatetraenoic acid (ETA), Heneicosapentanoic acid (HPA), Docosapentanoic acid (DPA), Clupanodonic acid, Tetracosapentaenoic acid and Tetracosahexaenoic acid (Nisinic acid) have gained importance since they have been evidenced for highly active nature.

Table: Presence of Omega-3 fatty acid (Linolenic acid) in different silkworm pupae oil

Source of oil	Percentage
Mulberry silkworm pupae	0.69
Tasar silkworm pupae	32.08
Eri silkworm pupae	20.95
Muga silkworm pupae	10.91

Recently, a study conducted by the authors on mulberry and non-mulberry silkworm pupae oils revealed identification of silkworm pupae oil as a new source of omega-3 fatty acids. The petroleum ether extraction of mulberry, tasar, eri and muga silkworms' pupae oils were carried out by employing maceration method. The volatile fraction and extracted mulberry and non-mulberry silkworm pupal oils were analyzed by Gas Chromatography-Mass Spectroscopy method and found for the presence of ALA. Among four analysed pupal oils, the most abundant constituent in the volatiles was tasar pupal oil followed by eri, muga and mulberry (Table). The study is, therefore, the first report on the chemical composition of all four pupal species of both mulberry and non-mulberry pupae oils.

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Extraction of silkworm pupal oil

Omega-3 fatty acids available in pupae oil can be utilized for human consumption by eating pupae or by taking supplements in the form of extracted pupae oil capsules similar to fish oil capsules. Further, pupae oil can also be refined by steam distillation or by filtering through activated charcoal or Fuller's Earth Method. Removal of the odour from the oil is not the problem; instead, the problem is related to the economics of the whole process. The oil extracted is brownish with fishy odour which can be cleared off by passing steam through the oil. The refined oil may be utilized as an alternative to edible oil and dalda.

Modern life-style of the people is characterized by lack of physical exercise, adulterated food stuffs and polluted air and water are associated with great quantity of free radicals generation, which leads to human health turbulences and even severe illnesses. In this context, science is facing the challenge of finding out the way to prevent diseases and slow the ageing process. One such a way, using Omega-3 fatty acids as food supplement will certainly help to enhance human health. For that reason, pupal oils of both mulberry and non-mulberry will play a major role, as they are a great source of omega-3 fatty acid. Pupal oils stand as a promising challenge for researchers for its efficacy against chronic diseases.

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