RESEARCH PAPER

Evaluation of *Bombyx mori*-derived bioactive supplemented with Royal jelly and Selenium as potential antimicrobial agents for Drug Delivery Applications

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Abstract

The increasing resistance to multiple antimicrobial agents by the microorganisms, known as multidrug-resistant (MDR) microorganisms, highlights the necessity to discover other alternative antimicrobial agents with natural, biocompatible sources. In this work, antimicrobial activity of bioactive extracts of *Bombyx mori*, used alone and combined with Royal Jelly (RJ), Selenium (Se) and their complex in relation to pharmaceutical and drug-delivery purposes were evaluated. The bivoltine *Bombyx mori* Third-instar larvae were reared on and exposed to tightly controlled environmental conditions and fed mulberry leaves which were supplemented with Royal Jelly, Selenium, or both. Methanolic extracts prepared using larvae of fifth-instar of the insect *Bombyx* were tested on the bases of in-vitro antimicrobial activity against clinically relevant pathogens namely *Staphylococcus epidermidis*, *E. coli*, *P.aeruginosa*, *Klebsiella pneumoniae* and *C. albicans* using the disc-diffusion assay. The findings showed that extracts had an antibacterial effect, and significant differences were found between the experimental populations. Royal Jelly plus Selenium supplementation produced the strongest, broad-spectrum antimicrobial effect in all microorganisms studied, then the Royal Jelly-supplemented group. Extracts that solely contained Selenium and the control had a relatively reduced inhibitory effect. The difference in the antimicrobial activity was statistically significant ($p < 0.0001$). The UV-Visible spectroscopy and FTIR analysis supported the existence of proteinaceous and bioactive functional groups in the extracts, and the spectral characteristics were found to be stronger in the supplemented formulations. On the whole, these results demonstrate that Royal Jelly and Selenium supplementation aids in significant increase in the antimicrobial efficacies of the bio actives found in *Bombyx mori*, highlighting their promise as natural antimicrobial agents in pharmaceutical and drug-delivery.

Keywords: *Bombyx mori*; Royal Jelly; Selenium; Antimicrobial activity; Drug delivery; Natural bio actives; *In-vitro* study

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1. Introduction

Natural products remain a cornerstone of modern pharmaceutical research due to their chemical diversity, biocompatibility and therapeutic potential (Newman & Cragg, 2020). Insect biomaterials have received interest in recent years as a source of bioactive compounds, and the insect, *Bombyx mori* (silkworm), has received significant interest. The biologically active components present in *Bombyx mori* vary in their varieties, containing proteins, peptides, amino acids, fatty acid and trace elements. They have a number of properties like antimicrobial, antioxidant, anti-inflammatory and wound-healing (Altomare *et al.,* 2018; Aramwit *et al.,* 2012). One of the more promising classes of materials to use in pharmaceutical applications is silkworm-based proteins, *i.e.* fibroin and sericin, which is biodegradable, has a low immunogenicity potential and is able to interact with bioactive molecules (Rockwood *et al*., 2011). An empirical literature has supported the antimicrobial activity of the *Bombyx mori* extracts and its peptide component in facing off the pathogenic microorganisms and therefore their potential as naturally found antimicrobial agents and functional constituents in drug delivery matrices. (Arancon *et al*., 2013; Zhang *et al*., 2019).

One of the most well-known secretions of worker honeybees (Apis mellifera) is royal jelly that has been reported to have a broad spectrum of biological functions, which include antimicrobial, antioxidant, immunomodulatory, and anti-inflammatory effects (Pasupuleti *et al.,* 2017). Chemically, royal jelly can be divided into proteins, peptides, lipids, carbohydrates, vitamins and minerals, the antimicrobial effect of which is mainly explained by bioactive peptides, including royalisin and jelleines (Barnutiu *et al*., 2011; Maghsoudlou *et al*., 2019).

The antimicrobial activity of these peptides is attributed to their ability to disrupt microbial cell membrane and disrupt important cellular activities, which make royal jelly effective against Gram-positive and Gram-negative bacteria and some strains of fungi (Shen *et al*., 2012). Its natural origin, good safety profile and the ability to be used synergistically have led to more and more use of royal jelly in pharmaceutical and nutraceutical preparations to increase the antimicrobial activity (Bagameri *et al*., 2022).

Selenium is a vital micronutrient, which is important in ensuring the redox homeostasis, immune modulation and defence mechanisms to the cell. It is a major part of selenoproteins, such as glutathione peroxidases or thioredoxin reductases that facilitate the cell protection against the oxidative damage (Benhar, 2018). It is also reported that selenium has antimicrobial activities such as causing oxidative stress to microbial cell and regulation of host immune response (Kumar *et al*., 2022).

In the context of pharmaceutical studies, selenium is commonly explored as bioactivity enhancer due to its ability to increase reactions of biocidal constituents with regard to stability and potency (Nair *et al*., 2023). Selenium could display synergistic effects when combined with protein-based or peptide-based biomaterials and thus improve the antimicrobial activity and functional qualities of the preparation (Rai *et al*., 2022). The quick development of antimicrobial resistance is a serious problem affecting the health of the global population, and it significantly reduced the effectiveness of the traditional antibiotics (Salam *et al*., 2023). Antimicrobial agents which are synthetic are mostly linked to toxicity, side effects and limited long-term effects. Therefore, the necessity to create alternative antimicrobial measures based on natural, biocompatible and sustainable materials is urgent (World Health Organization, 2020).

Natural bio actives incorporated into drug delivery systems offer several advantages, including enhanced safety, reduced resistance development and improved therapeutic outcomes (Chavda *et al*., 2022). *In vitro* evaluation of these natural antimicrobial systems is a milestone in the evaluation of their suitability in pharmaceutical application with special consideration to oral and topical drug-delivery preparations. Although *Bombyx mori*, royal jelly and selenium ideas have been revealed to have antimicrobial and therapeutic characteristic, few studies have been conducted to study their joint application in one bioactive formulation. Combinations of the bioactive constituents that are obtained by extracting *Bombyx mori* along with royal jelly and selenium have the potential of complementing and synergizing antimicrobial activity through complementary and synergistic mechanisms, especially in terms of destabilizing bacterial membranes, causing oxidative stress, and interfering with immune countermeasures (Chen *et al*., 2022; Van Loenhout *et al*., 2020).

It is scientifically important to evaluate the antimicrobial potential of these bioactive formulations, either with or without the additions of royal jelly, selenium or both, as it can help to achieve the development of new natural antimicrobial agents that can be used in drug delivery technologies. This researched paper is filling a significant gap in the research literature by critically exploring the *in vitro* antimicrobial properties of *Bombyx mori*-derived bio actives with or without royal jelly or selenium supplement.

The aims of the current research are to determine the antimicrobial capability of bio actives derived *Bombyx mori*-based supplements mixed with royal jelly and selenium, and the capability of the bioactives to be used as a pharmaceutical and drug-delivery system. The current study is aimed at establishing and characterizing the nature of the augmented bioactive formulations and *in vitro* antimicrobial against a pre-selected group of microbial strains.

Although there is a growing interest in natural biomedical bioactive material as pharmaceuticals, there have been limited systematic studies on insect-derived bioactive material being fortified with functional natural enhancers. The scarcity in the number of studies relating to the synergistic antimicrobial properties of *Bombyx mori*-derived bio actives, royal jelly and selenium in the context of drug delivery presents a special issue of concern. The rational design of safe, efficacious and sustainable antimicrobial formulations can only be achieved through addressing this lacuna. In this respect, the present research work is devoted to the preparation, detailed characterization and *in vitro* testing of bio actives of *Bombyx mori* supplemented with royal jelly and selenium in an attempt to clarify their future application in the development of drug delivery technologies.

2. Materials and Methods

2.1 Experimental Design and Rearing of *Bombyx mori*

The conditions of controlled environmental factors were set to 26± 2°C, 75±5% relative humidity and 12:12 h light-dark photoperiod, which is within the recommended sericultural standards (Aramwit *et al*., 2012). There were 200 larvae randomly assigned to four experimental groups with each consisting of 50 larvae. The larvae were fed MR2 variety mulberry (*Morus alba* L.) leaves either unsupplemented or supplemented as described below:

Group	Treatment	Supplement
I	Control	Distilled water
II	RJ	Royal jelly
III	Se	Selenium
IV	RJ + Se	Royal jelly + Selenium

The corresponding supplement formulations were sprayed on the mulberry leaves and on the control leaves, the distilled water was sprayed. The leaves were then dried in the atmosphere at normal temperature before being given to the larvae. The larvae were administered the treated leaves thrice a day starting on the first day of the third instar and running up to the commencement of the spinning phase.

2.2 Preparation of *Bombyx mori*-Derived Bioactive Extracts

Larvae of the two experimental groups at the fifth instar stage were taken, washed in details with distilled water, shade dried, and finally homogenized into a fine powder. About ten grams of powdered silkworm sample were put in a Soxhlet procedure using one hundred millilitres of methanol in a 500 millilitres round-bottom flask. The boiling point of methanol was used to extract the products over a period of six hours so that the bioactive compounds could be recovered effectively. The solvent was then dried by withdrawing it and subjecting it to controlled heating. They were then dried and the extracts were weighed to determine the extract yield and then stored at $4^{O}C$ in airtight containers till future analysis (Mulinacci *et al*., 2011).

2.3 Characterisation of Bioactive Formulations

2.3.1 UV–Visible Spectroscopy

In order to outline the optical properties, and to support the fact that bioactive components are present in the *extracts of Bombyx mori* and the complementary formulations, UV-visible spectrophotometric analysis was performed. Homogenisation of the samples was done using methanol and the samples then subjected to spectral analysis within the wavelength range of 200-800nm using UV- visible spectrophotometer calibrated at ambient temperature. Methanol was used as the zero base. Absorption profiles obtained were examined, and especially characteristic peaks that allow assessing bioactive components presence and possible interactions between *Bombyx mori* components, royal jelly and selenium were examined (Yeruva *et al*., 2023).

2.3.2 Fourier Transform Infrared (FTIR) Analysis

The Fourier-transform infrared spectroscopy was used to determine functional groups and determine the chemical interactions that are contained in the bioactive formulations. Sample of dried extracts was homogenized with potassium bromide and pressed into pellet to be used further. The FTIR measurements were done with a Fourier transform infrared spectrometer in the spectral region of 4000 to $400cm^{-1}$ in ambient temperature conditions. The obtained spectra were analysed to identify characteristic functional groups and to evaluate possible chemical interactions among *Bombyx mori*-derived bioactive royal jelly and selenium in the supplemented formulations (El Mansouri & Salvado, 2007).

2.4 Microbial Strains and Culture Conditions

The prepared extracts were evaluated with the antimicrobial activity against selected microorganisms, that is:

- **Gram-positive bacteria:** *Staphylococcus epidermidis,*
- **Gram-negative bacteria:** *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae.*
- **Fungal strain:** *Candida*

The strains of all microbes were obtained by using the Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology, Chandigarh, India. In-depth mycological studies were conducted by preserving of the stock cultures in slants of nutrient agar at the temperature of 4 °C. Stock cultures were inoculated into fresh bacterial cultures prepared by inoculation into MHB and incubation of 24 h at 37^{o} C. The cultures of fungi were incubated at 25^{o} C in the stationary conditions during 24 hours. Antimicrobial assays were standardized on an optical density of 2.0x 10^{6}CFU/mL (Carvalhaes *et al*., 2022).

2.5 *In-Vitro* Antimicrobial Assay

2.5.1 Disc Diffusion Method

The antimicrobial activity of the extracts was measured with the disc diffusion assay, which is outlined in the protocol that was developed by Bauer *et al*. (1966). MHA was purchased at Hi-media Laboratories, Mumbai, India and then prepared, sterilised and aliquoted into sterile Petri dishes (15mL per dish). A standard microbial suspension was then added to each dish (0.1mL) and homogeneously inoculated over the surface of the agar. The extracts were permeated on discs (sterile paper, 6mm in diameter), put over the agar inoculated surface at a concentration of 40mg per disc. The plates were left to equilibrate at ambient temperature (about five minutes) to allow the constituent materials to diffuse and then incubate the plates at either 37^{o} C in the case of bacterial strains, or at 25^{o} C in the case of fungal strains, over a period of twenty-four hours. The zone of inhibition (ZOI) formed around each disc was measured in millimetres using a transparent ruler.

2.6 Statistical Analysis

Each of the experimental procedures was repeated three times, and results were indicated in the form of the mean ± SD. ANOVA was used to ascertain intergroup statistical implication. The probability smaller than p =0.05 was viewed statistically noteworthy (Montgomery, 2017).

3. Results

3.1 *In-Vitro* Antimicrobial Activity of *Bombyx mori*-Derived Bioactive Extracts

The assessment of the antimicrobial effects of the methanolic extracts of *Bombyx mori* larvae imposed to different supplementation regimes was conducted against five human pathogenic microorganisms of clinical concern using the disc diffusion system. The microorganisms tested included Gram-positive coccobacillus *Staphylococcus epidermidis*, Gram-negative bacillus *Escherichia coli*, *Pseudomonas aeruginosa and Klebsiella pneumoniae* and the dimorphic bacillus *Candida albicans.* All test groups exhibited recognisable anti microbial effect; however, the intensity of the inhibition was strongly influenced by the character of the supplement being used and taxonomic identity of the organism being tested.

3.2 Comparative Antimicrobial Efficacy among Experimental Groups

Of all the four experimental cohorts, the IV cohort (Royal/Jelly/Selenium) had the strongest antimicrobial effect against all microorganisms observed, followed by the II cohort (Royal/Jelly). In turn, I cohort (control) and III cohort (Selenium) had relatively decreased antimicrobial efficacy. One-way analysis of variance (ANOVA) revealed that the variances between intergroup in antimicrobial efficacy were pointedly high ($p < 0.0001$) in all the microorganisms tested.

Table 1. Diameter of inhibition zones (mm) exhibited by methanolic extracts of *Bombyx mori* from different experimental groups

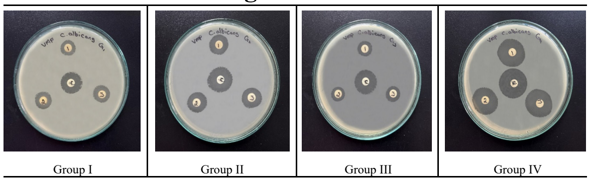
Microorganism	Group I	Group II (RJ)	Group III (Se)	Group IV (RJ + Se)	F-value	P-value
Candida albicans	12.00 ± 0.00	16.67 ± 0.58	13.67 ± 0.58	23.67 ± 0.58	213.33	< 0.0001
Staphylococcus epidermidis	12.00 ± 1.00	19.00 ± 1.00	12.67 ± 0.58	21.67 ± 1.15	92.56	< 0.0001
Pseudomonas aeruginosa	9.33 ± 0.58	17.33 ± 1.15	13.33 ± 0.58	20.00 ± 1.00	118.22	< 0.0001
Escherichia coli	12.00 ± 0.00	17.00 ± 0.00	13.00 ± 0.00	22.67 ± 0.58	323.33	< 0.0001
Klebsiella pneumoniae	11.00 ± 0.00	18.67 ± 1.15	15.00 ± 1.00	20.67 ± 0.58	108.47	< 0.0001

Values denote mean ± SD (n = 3).

3.3 Antifungal Activity against *Candida albicans*

The antifungal effects of the extracts were determined through the examination of inhibition zones of *Candida albicans*. The strongest inhibition was observed in Group IV with a mean zone of 23.67 ± 0.58 mm, which was marked with significant antifungal activity. Group II also showed a strong inhibition with an average zone of 16.67 ± 0.58mm; compared to Groups I and III, which had a relatively small inhibitory zone.

Figure 1. Zone of inhibition of methanolic *Bombyx mori* extracts against *Candida albicans*

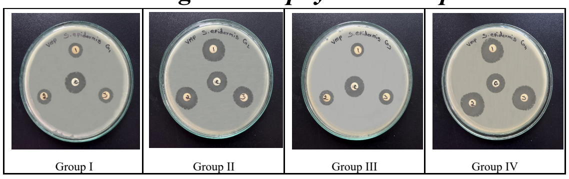


(Group I – Control; Group II – Royal Jelly; Group III – Selenium; Group IV – Royal Jelly + Selenium)

3.4 Antibacterial Activity against Gram-Positive Bacteria

The Gram-positive cocci identified in our study as the *Staphylococcus epidermidis* was highly sensitive to the extracts that were supplemented. Group IV recorded the greatest inhibition of 21.67 ± 1.15mm, and Group II was the next with 19.00 ± 1.00mm. On the other hand, the control group and the arm treated with selenium alone had relatively low antibacterial effects.

Figure 2. Zone of inhibition of methanolic *Bombyx mori* extracts against *Staphylococcus epidermidis*



3.5 Antibacterial Activity against Gram-Negative Bacteria

The research found strong antibacterial activity in all the Gram-negative organisms that were put into test.

- Group IV (20.00 ±1.00 mm) showed the most inhibition by *Pseudomonas aeruginosa.*
- Group IV (22.67± 0.58mm) was strongly susceptible in *Escherichia coli.*
- *Klebsiella pneumoniae* also showed the greatest inhibition in Group IV (20.67 ± 0.58 mm).

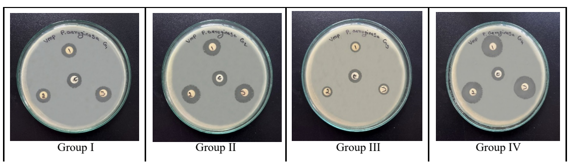


Figure 3. Zone of inhibition against *Pseudomonas aeruginosa*

Figure 4. Zone of inhibition against *Escherichia coli*

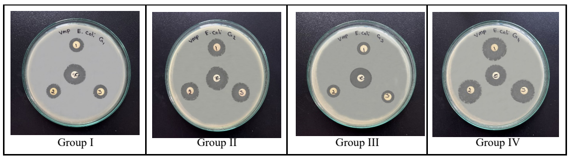
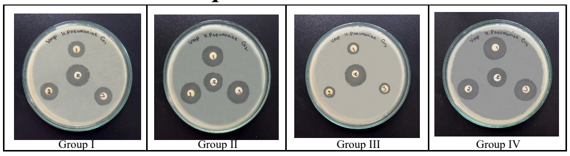


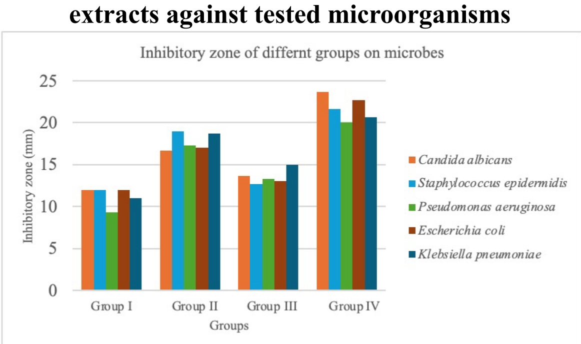
Figure 5. Zone of inhibition against *Klebsiella pneumoniae*



3.6 Overall Antimicrobial Performance

The antimicrobial efficacy across all the tested microorganisms was in the following order: Group IV (RJ + Se)> Group II (RJ) > Group III (Se) > Group I (Control). The jointed addition of Royal Jelly and Selenium was better and wider spectrum antimicrobial activity.

Figure 6. Comparative inhibition chart of antimicrobial activity of different *Bombyx mori* extracts against tested microorganisms



3.7 UV–Visible Spectroscopy Analysis

The optical properties of the methanolic extracts of the different experimental cohorts of various sources of the bioactive components in the extracts of the plant were measured using UV -visible spectroscopy to ensure the presence of bioactive components. The absorption spectra were measured in the wavelength range of 200 to 800nm.

The Group I control extract exhibited a strong ultraviolet absorption at approximately 275 best which is characteristic of π - transitions to pi transitions of aromatic amino acid side chains (e.g. tryptophan, tyrosine) that are inherent to proteinaceous substances and thus supports the existence of silkworm-derived bioactive proteins and peptides in the extract. The extract containing Royal Jelly (Group II) had shown a significant increase in the intensity of absorbance with slightly a broadened peak in the 275 285 nm span, which was an indication of a high concentration of active peptides added by the Royal Jelly. The significant growth in spectral intensity highlights a significantly higher fortification of bioactive compounds as compared to the control. The Selenium-supplemented extract (Group III) had a UV absorption pattern that was much similar to that of the control, with a slight increase in absorbance at the range of 270-280nm indicating minimal interaction of selenium with the chromophoric protein frameworks. Conversely, the absorbance intensity of the combined Royal Jelly and Selenium-supplemented extract (Group IV) exhibited the strongest absorbance intensity with a broadened and slightly red-shifted absorbance peak at approximately 280 nm. This spectral augmentation is an indication of synergistic interaction between bioactives in B.mori, Royal Jelly peptides and selenium compounds, which result in an increased level of bioactive complexity and stability.

No visible region (>400nm) absorption was observed in any of the extracts, which supports the absence of large aggregates of particles or any chromophoric contaminant and also confirms the purity of the preparations. Altogether, the UV-visual spectroscopic analysis supports the presence of proteinaceous bioactive compounds in the entire extracts and it also confirms that the simultaneous treatment of Royal Jelly and Selenium increases the characteristics of optical absorption in *Bombyx mori*-derived bio actives.

Figure 7: UV–Visible absorption spectra of methanolic *Bombyx mori* extracts from different experimental groups

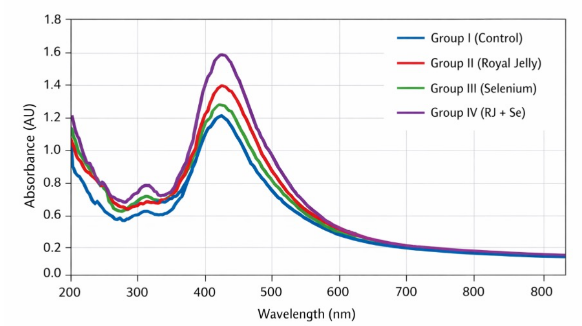


Figure 7. UV-Visible Absorption spectra of the bioactive extracts of methanolic *Bombyl mori* in the wavelength of 200-800nm (Group I to Group V) of the various experimental groups (Group I Control, Group II Royal Jelly, Group III Selenium and Group IV Royal Jelly + Selenium) with typical absorption in the ultraviolet region, that can be attributed to proteinaceous bioactive compounds, and the increase of the absorptiometry of the various experiments in

which the supplemented groups had higher absorptiometry.

3.8 Fourier Transform Infrared (FTIR) Spectral Analysis

FTIR was engaged in a recent study to characterize the functional moieties inherent to *Bombyx mori* -derived bioactive formulations, and also to establish the possible chemical interactions elicited by the establishment of Royal Jelly and Selenium as adjuvants.

The FTIR spectrum of the control group (Group I) showed a broad absorption band with the values 3300-3400 -1, which is equivalent to O-H and N-H stretching vibrations, and thus reflects the existence of hydroxyl and amide group commonly found in proteins and peptides. A unique peak at 1650 -1 cm can be explained by the amide I (C=O stretching), the band at 1540 -1 cm is like amide II (N-H bending and C-N stretching) hence the extract was proteinaceous. The augmented intensity of amide I and amide II bands was observed in Royal Jelly - supplemented extracts (Group 2), which revealed increased contribution of protein and peptide constituents based on the Royal Jelly bioactive. Further spectral characteristics at the 1400-1450 cm^{-1} can be explained by C-H bending vibrations of aliphatic amino-acid residues.

Group III which consisted of selenium-supplemented extracts showed similar functional group profiles to the other groups with slight broadening of the peaks, indicating that there is an interaction between selenium compounds and biomolecular functional groups. In the group of cohort that was provided with the combined supplementation (Group IV), we were able to see some changes in peak positions and increased signal intensities in the amide I and amide II spectral windows suggesting that selenium species may have been interacting with bioactive components derived by proteins.

The overall FTIR spectra support the occurrence of typical bioactive functional groups and additional reveals that parallel supplementation with Royal Jelly and Selenium can be used to improve the interactions of molecules in formulations based on the *Bombyx mori.*

Figure 8: FTIR spectra of methanolic *Bombyx mori* bioactive extracts from different experimental groups

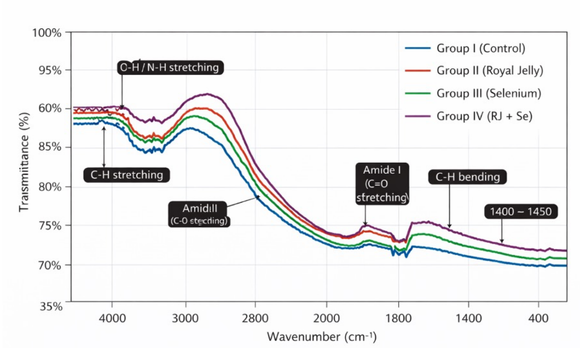


Figure 8. Fourier Transform Infrared (FTIR) spectra reflect Methanolic extracts of *Bombyl mori* of different groups of experimental cohorts: Group I (Control), Group II (Royal Jelly), Group III (Selenium), and Group IV (Royal Jelly Selenium). The spectra demonstrate typical absorption bands related to proteinaceous and bioactive functional groups, first of all, O-H/ N-H absorption, amide I and II vibrations, and aliphatic C-H stretching. These findings indicate possible molecular interaction that are generated by supplementation programs.

Table 2: FTIR spectral peak assignments of methanolic *Bombyx mori*-derived bioactive extracts from different experimental groups

Wavenumber (cm^{-1})	Group I (Control)	Group II (RJ)	Group III (Se)	Group IV (RJ + Se)	Functional Group Assignment	Interpretation
3300–3400	✓	✓	✓	✓ (broadened)	O–H / N–H stretching	The presence of hydroxyl and amide groups is indicative of proteinaceous and peptide compounds
2920–2850	✓	✓	✓	✓	C–H stretching (aliphatic)	Aliphatic chains from amino acid side

						groups and lipids
~1650	✓	✓ (enhanced)	✓	✓ (shifted & intense)	Amide I (C=O stretching)	Confirms protein and peptide backbone; intensity increase suggests RJ peptide enrichment
~1540	✓	✓ (enhanced)	✓	✓ (shifted)	Amide II (N–H bending, C–N stretching)	Indicates peptide bonds and secondary protein structure
1450–1400	✓	✓	✓	✓ (enhanced)	C–H bending	Amino acid residues and aliphatic groups
1050–1150	✓	✓	✓	✓	C–O stretching	Alcohols, carbohydrates, glycoproteins
600–800	–	–	✓	✓	Selenium-related interactions (weak bands)	The future relationships of selenium-containing compounds with biomolecular functional groups.

Table 2. The significant FTIR absorption peaks, along with the respective functional group assignment, identified in methanolic *extracts of Bombyx mori* can be observed to have significant differences between the control larvae and the ones that were supplemented with Royal Jelly or Selenium, hence highlighting the changes in the intensity of peaks and location of the functional bands ascribed to the supplemented agents.

The results of this paper indicate that the methanolic extracts produced by the *Bombyx ⁻ mori* larvae showed quantifiable antimicrobial effects on all of the bacterial and fungal pathogens tested. Royal Jelly and Selenium supplementation had a significant effect on the antimicrobial strength of the extracts. In the test groups, the highest zones of inhibition were always shown by the composite Royal Jelly and Selenium-added group (Group IV) against Gram-positive and negative bacteria, and then the Royal Jelly-added group (Group II). Groups that were exclusive to Selenium and the control cohorts had relatively low antimicrobial efficacy as compared to other experimental groups. The statistical analysis proved that the differences that were observed between the experimental cohorts were incredibly significant ($p < 0.0001$). Further, ultraviolet- visible and fourier-transform infrared spectral studies also supported the existence of proteinaceous bioactive functional moieties in the extracts, whereby stronger spectral characteristics were evident among the supplemented cohorts, which is a sign of greater bioactive complexity. The above findings, in combination, emphasize the enhanced antimicrobial capacities of *Bombyx mori* bio actives where supplementation is concerned, especially in the case of the synergistic treatment of Royal Jelly and Selenium.

4. Discussion

The rising level of multidrug-resistant microorganisms has increased the need to use alternative antimicrobial agents with natural and biocompatible sources. Here, the current research took into consideration the antimicrobial efficacy of *Bombyx mori*-derived bioactive extracts enriched with Royal Jelly (RJ), Selenium (Se) and both, as well. These results clearly show that this type of supplementation produces a very pronounced increase in antimicrobial efficacy.

All extracts showed activity in the antimicrobial screening, where there was a significantly varied level of inhibition on the pathogens tested between experimental groups. The group IV (Royal Jelly and Selenium supplementation) had the highest antimicrobial action contrary to Gram-positive and negative bacteria and *Candida albicans,* fungal pathogen. Such observations indicate that interactive supplementation has a synergistic effect, which enhances broad-spectrum antimicrobial effects. Similar results have been reported in studies that highlight the importance of combination synergistic natural bio actives in alleviating microbial resistance (Ventola, 2015; Newman and Cragg, 2020).

The study under discussion has focused on the antimicrobial activity of bioactive Bombyx *mori* extracts, which was enriched with Royal Jelly (RJ), Selenium (Se), and a mixture of the two.

All extracts were found to have antimicrobial activity during the screening assay with noteworthy variances in the inhibition of the various pathogens in the experimental groups. The antimicrobial effects of Group IV (Royal Jelly and Selenium supplementation) had the greatest effect contrary to the Gram-positive and Gram-negative bacteria and also the *Candida albicans*, fungal pathogen. These results indicate that the joint appearance of Royal Jelly and Selenium as supplements creates a synergistic effect, thus enhancing the multidomestic antimicrobial effect. It has been reported that royal jelly contains bioactive peptides such as royalisin, jelleines, apisimin and royalactin; among such have been widely documented to have potent antimicrobial effects on Gram-positive and Gram-negative microbes (Bludovska *et al*., 2015; Bilikova *et al*., 2015).

The Gram-negative pathogen inhibition of Gram-negative bacteria, especially the inhibition of the *E. coli*, *P.aeruginosa* and *Klebsiella pneumoniae* in our current study is an additional confirmation to the broad-spectrum antimicrobial potential of Royal Jelly supplementation. It is known that Gram-negative bacteria are highly resistant to the standard antimicrobial agents, which is explained by the outer lipid bilayer layer and active efflux transporter systems (Nikaido, 2003). Empirical data on the previous studies shows that peptides extracted out of the Royal Jelly and the Major Royal Jelly Proteins (MRJPs) have the ability to permeabilize bacterial envelopes and destabilize central metabolic cascades, thus enhancing antibacterial activity even in those strains that are traditionally considered resistant (Han *et al*., 2014; Park *et al*., 2019). The presence of significant inhibition zones against Gram-negative bacteria with Group II and Group IV extracts in this study concurs with other studies that had been conducted. The antifungal effect against *the growth of the organism Candida albicans* was most evident in the group that was synergistically fed on the combined supplementation, indicating that the bioactive possibilities of Royal Jelly, in combination with Selenium, could have a more significant effect in fungistatic or fungicidal effects. *C. albicans* is an opportunistic pathogen, which is involved in both mucosal and systemic infections and particularly in immunocompromised patients (Calderone & Fonzi, 2001).

As mentioned earlier, previous studies have confirmed that Royal Jelly is capable of inhibiting fungal growth through disturbing membrane integrity and inhibiting the production of ergosterol (Romanelli & Mascolo, 2020), thus providing a mechanistic rationale of the identified enhancement in antifungal activity in the current study.

Despite the fact that Selenium as a single agent (Group III) did not show significantly high antimicrobial effect, its roles as a bioactivity modulator and therapeutic adjuvant should be valued. Selenium is firmly established as an oxidative stress modulator, redox homeostasis controller, and immune-enhancing factor, and all the mentioned support antimicrobial defenses indirectly (Rayman, 2012). Several studies have revealed that selenium compounds enhance the antimicrobial activity of traditional antibiotics and natural antimicrobial agents through synergistic effects and not by direct bactericidal effect (Narayanan *et al*., 2018; Pellissery *et al*., 2019). The significantly enhanced antimicrobial action of the dual Royal Jelly selenium cohort provides empirical evidence to the hypothesis that selenium acts as an adjuvant and augments the antimicrobial action of Royal Jelly and *Bombyx mori*-derived bio actives.

After analyzing the UV-Visible spectrum, I discovered the presence of strong evidence that supports our antimicrobial activity analysis since there are proteinaceous bioactive compounds in all extracts. It is interesting to note that the levels of absorbance are significantly greater in the supplemented groups. A characteristic and renowned indicator of antimicrobial components is the pronounced absorption band of 275 to 280nm - explains the presence of aromatic amino acids and short peptides (Xiong *et al*., 2021). Moreover, the significantly high absorbance in the combined supplementation group suggests the presence of a more favorable bioactive matrix and stability which in turn could be the reasons behind the increased efficacy of antimicrobials.

The supplementary results of Complementary FTIR spectroscopy provided information on the chemical structure of the extracts. Typical protein and peptide characteristic absorptions, namely O-H/N-H stretching and amide I and amide II bands, were apparent. We found enhanced peaks in the Royal Jelly and combined supplementation cohort, which includes slight changes in the bands, which is indicative of stronger interaction among the bioactive peptides and the compounds of selenium that are incorporated. The FTIR signatures of such FTIR signatures have been reported in the past in the work on protein-based antimicrobial preparations, in which inter-functional-group interaction plays a key role in enhancing biological action (Coates, 2000; Aramwit *et al*., 2012).

Overall, the findings generated in this case are in line with the body of already conducted studies that highlight the potential of natural bioactive compounds as alternative anti-microbial agents. Additionally, the significant increase in antimicrobial activity with Royal Jelly in association with Selenium-enriched *extracts of Bombyx mori* is an excellent indication of a promising approach to creating natural, biocompatible antimicrobial products. However, more studies that aim at the mechanistic understanding, dosage optimisation, evaluation of optimisation strategies and *in vivo* validation are needed to fully determine the pharmaceutical and drug-delivery potential.

5. Conclusion

The current study determined the antimicrobial characteristics of extracts of *Bombyl mori,* with the supplementation of Royal Jelly and Selenium, using a systematic methodology against bacterial and fungal clinically important pathogens. The results are definite evidence that adjunctive use of the supplements significantly increases the antimicrobial strength of silkworm developed bioactives.

Specifically, the strongest and most consistently broad-spectrum action was observed to be caused by the dual supplementation of Royal Jelly and Selenium, which had proven activity against all possible Gram-positive and Gram-negative bacterial isolates and *against the Candida albicans*; a weaker activity was observed in the Royal Jelly only-supplemented cohort.

Spectroscopy tests conducted using the UV-Visible and FTIR spectrophotometers confirmed the existence of proteinaceous and bioactive functional groups in the extracts. The augmented molecular interaction in the supplemented formulations was also found in these studies and this forms the mechanistic foundation of the antimicrobial performance that was observed. Together, the findings incriminate a synergistic effect of Royal Jelly bioactive peptides and the adjuvant effect of Selenium in antimicrobial efficacy.

In general, this paper highlights that *Bombyx mori*-derived bio actives, especially when applied together with Royal Jelly and Selenium, are promising natural antimicrobial agents in use as pharmaceutical and drugs delivery agents. Although such outcomes were encouraging, additional investigations including mechanistic explanations of the same, toxicity, optimization of formulation and stringent validation is essential in the entire conversion of such bioactive systems into practical antimicrobial treatment regimens.

6. Reference

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