Synergistic Amelioration of Imidacloprid-Induced Infertility by Vitex agnus-castus and Nigella sativa in Female Rats: Mechanistic Evidence of Hormonal and Reproductive Restoration

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Research Article

Keywords: Hormonal imbalance, Imidacloprid, Infertility, Nigella sativa, Phytotherapy, Reproductive toxicity, Vitex agnus-castus

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Synergistic Amelioration of Imidacloprid-Induced Infertility by *Vitex agnus-castus* and *Nigella sativa* in Female Rats: Mechanistic Evidence of Hormonal and Reproductive Restoration

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ABSTRACT

Background:

Imidacloprid, a widely used neonicotinoid pesticide, is associated with endocrine disruption and reproductive toxicity in females. Phytotherapeutics such as *Vitex agnus-castus* and *Nigella sativa* have shown promising effects in fertility regulation and hormonal modulation. This study aimed to assess their therapeutic potential, individually and in combination, against Imidacloprid-induced infertility in female Wistar rats.

Methods:

A total of 48 Wistar albino rats (36 females and 12 males) were divided into seven groups (n = 6). Infertility was induced using Imidacloprid. Test groups were treated with *Vitex agnus-castus*, *Nigella sativa*, their combination, or standard clomiphene citrate for 28 days following induction. Hormonal assays (LH, FSH, estrogen, progesterone), fertility index, pup birth rate, and histopathological evaluation of ovaries and uterus were performed.

Results:

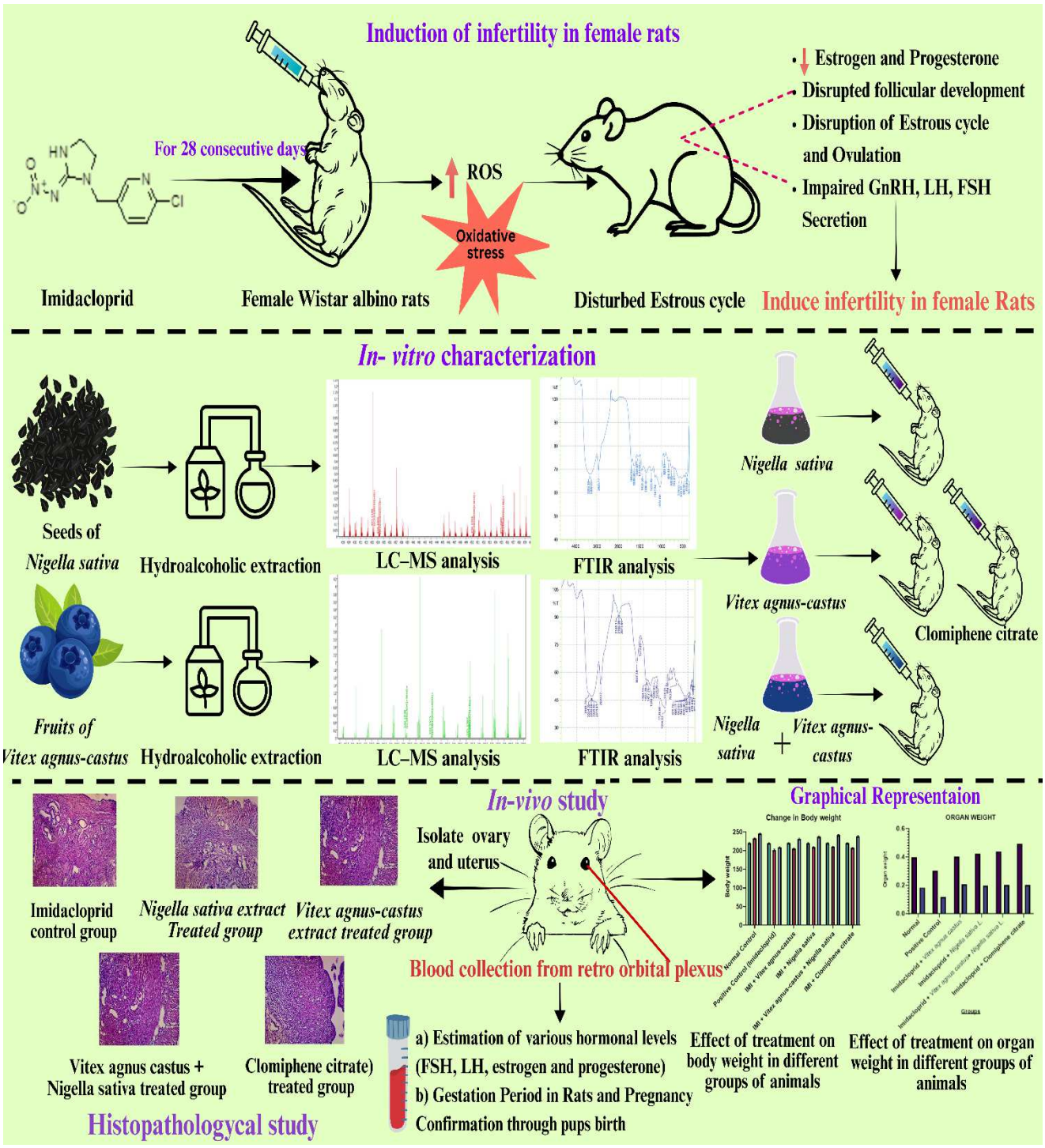
Imidacloprid significantly reduced fertility index, altered reproductive hormones, and caused ovarian and uterine structural damage. Treatment with *Vitex agnus-castus* and *Nigella sativa* individually improved hormonal balance, fertility index, and histopathological architecture. Combination therapy demonstrated superior efficacy compared to individual treatments, approaching the effects of clomiphene citrate.

Conclusion:

Vitex agnus-castus and *Nigella sativa* confer protection against Imidacloprid-induced infertility through endocrine modulation and restoration of reproductive tissue integrity. Combination therapy exhibited synergistic effects, suggesting potential as a phytotherapeutic strategy for managing pesticide-induced female infertility.

Keywords:

Hormonal imbalance, Imidacloprid, Infertility, *Nigella sativa*, Phytotherapy,Reproductive toxicity, *Vitex agnus-castus*.



Graphical abstract

HIGHLIGHTS

- ✓ Imidacloprid, a neonicotinoid pesticide, induced significant infertility and hormonal imbalance in female Wistar rats.
- ✓ *Vitex agnus-castus* and *Nigella sativa* seed extracts demonstrated protective effects against pesticide-induced reproductive toxicity.
- ✓ Combination therapy of *Vitex agnus-castus* and *Nigella sativa* exhibited synergistic efficacy compared to individual treatments.
- ✓ Restoration of serum reproductive hormones (LH, FSH, estrogen, progesterone) confirmed endocrine modulation.
- ✓ Improvement in fertility index and pup birth rate validated reproductive restoration.
- ✓ Histopathological analysis revealed reversal of ovarian and uterine structural damages in treated groups.
- ✓ Clomiphene citrate (standard drug) validated the experimental model and therapeutic comparison.
- ✓ Findings suggest phytotherapeutic potential of *Vitex agnus-castus* and *Nigella sativa* in mitigating pesticide-induced female infertility.

1. INTRODUCTION

Infertility is a major global health concern, affecting nearly 10–15% of couples of reproductive ages and exerting significant psychosocial and economic impacts.[1] Recent estimates by the World Health Organization indicate that approximately one in six adults (17.5%) worldwide experience infertility during their lifetime, with prevalence comparable across high-, middle-, and low-income countries.[2] The Global Burden of Disease (GBD 2021) study reported that more than 110 million women were affected, reflecting a 21.9% increase in prevalence since 1990.[3] In India, the demographic burden is particularly alarming, with the country ranking highest globally in infertility cases and disability-adjusted life years (DALYs).[4] National surveys suggest that nearly 8% of Indian couples experience primary or secondary infertility, highlighting an urgent reproductive health challenge.[5]Environmental toxicants, especially endocrine-disrupting chemicals, play a pivotal role in declining fertility trends.[6] Among them, Imidacloprid, a widely used neonicotinoid pesticide, has been shown to induce oxidative stress, disrupt ovarian folliculogenesis, and alter reproductive hormone levels such as luteinizing hormone (LH), follicle-stimulating hormone (FSH), estrogen, and progesterone.[7] Prolonged exposure leads to impaired fertility, irregular estrous cycles, and histopathological changes in ovarian and uterine tissues. Phytotherapeutic agents with hormonal modulatory and antioxidant potential offer promising alternatives to conventional therapy. *Vitex agnus-castus* (chaste tree) has demonstrated dopaminergic and phytoestrogenic actions, normalizing gonadotropin release and supporting ovarian function. [8,9]*Nigella sativa* (black cumin), rich in thymoquinone, possesses antioxidant, anti-inflammatory, and endocrine-regulatory properties that improve reproductive outcomes.[10] Despite individual evidence, their combined therapeutic efficacy remains underexplored.The present study investigates the protective effects of *Vitex agnus-castus* and *Nigella sativa*, individually and in combination, against Imidacloprid-induced infertility in female Wistar rats, with emphasis on hormonal restoration, fertility outcomes, and histopathological changes.

2. MATERIAL AND METHODS

2.1. Chemicals and Plant materials

Imidacloprid (pesticide, analytical grade) was obtained from a certified supplier. Clomiphene citrate was used as the standard reference drug. The authenticated herbal extracts of *Vitex agnus-castus* and *Nigella sativa* were procured from Kshipra Biotech Pvt. Ltd., while the standard reference drug Clomiphene citrate was purchased from a licensed regional medical store. All other chemicals and reagents used in the study were of analytical grade and were supplied by the Department of Pharmacology, Columbia Institute of Pharmacy, Raipur (C.G.), India.

2.2.Phytochemical analysis of *Vitex agnus castus* and *Nigella sativa* by LCMS method [11,12]

The hydroalcoholic extracts of *Vitex agnus-castus* fruits and *Nigella sativa* seeds were subjected to phytochemical characterization using Liquid Chromatography–Mass Spectrometry (LC-MS). The analysis was performed on a high-performance LC system coupled with a mass spectrometer equipped with an electrospray ionization (ESI) source. Chromatographic separation was achieved on a reverse-phase C18 column (150 × 4.6 mm, 5 µm particle size) maintained at 30 °C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile with 0.1% formic acid), with a gradient elution program (5% B to 95% B over 30 min) at a flow rate of 0.5 mL/min. The injection volume was 10 µL, and detection was performed at 254 nm.

Mass spectrometric conditions were optimized as follows: ionization mode was operated in both positive and negative ESI, capillary voltage 4.5 kV, nebulizer gas (N_2) pressure 30 psi, drying gas flow 10 L/min, and desolvation temperature 350 °C. The scan range was set from m/z 100-1000. Calibration of the mass spectrometer was carried out using sodium formate solution.

Compounds were identified by comparing their retention times, accurate mass values, and fragmentation patterns with available literature data and spectral libraries. The analysis enabled the detection of flavonoids, phenolic acids, lignans, terpenoids, alkaloids, and quinones, which represent the major bioactive constituents of *Vitex agnus-castus* and *Nigella sativa*.

2.3.Phytochemical analysis of *Vitex agnus castus* and *Nigella sativa* by FTIR method[13,14]

The hydroalcoholic extracts of *Vitex agnus-castus* fruits and *Nigella sativa* seeds were subjected to Fourier Transform Infrared Spectroscopy (FTIR) for functional group characterization. The dried extracts were finely powdered and mixed with spectroscopic

grade potassium bromide (KBr) in the ratio of 1:100 (sample: KBr). The mixture was compressed into thin transparent pellets using a hydraulic press under vacuum.

FTIR spectra were recorded using an FTIR spectrophotometer (Shimadzu, Japan) in the mid-infrared region of 4000-400 cm^{-1} at a resolution of 4 cm^{-1}, with 32 scans per sample to improve the signal-to-noise ratio. A background spectrum of pure KBr pellet was first recorded under identical conditions and automatically subtracted from the sample spectrum to eliminate atmospheric and instrumental interference.

The characteristic absorption bands obtained were interpreted to identify major functional groups such as hydroxyl (-OH), carbonyl (C=O), alkene (C=C), amine (-NH), ether (C-O-C), and aromatic structures. These functional groups were correlated with the presence of phytochemical classes including flavonoids, phenolics, terpenoids, alkaloids, and fatty acids, thereby providing preliminary evidence of bioactive constituents in the extracts of *Vitex agnus-castus* and *Nigella sativa*.

2.4. Experimental Animals

A total of 48 healthy Wistar albino rats (36 females and 12 males; female-to-male ratio 3:1), weighing between 180-200 ± 10 g, were procured from the Institutional Animal House Facility, Columbia Institute of Pharmacy, Raipur, Chhattisgarh (Registration No.: 1321/PO/ReBi/S/10 CPCSEA, dated 22/10/2014). Prior to experimentation, the animals were acclimatized for 14 days under standard laboratory conditions (temperature 22 ± 2 °C, relative humidity 50–60%, and a 12 h light/dark cycle) with free access to a standard pellet diet and water *ad libitum*.[15] The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC Approval No.: CIP/IAEC/2025/246), in accordance with CPCSEA guidelines for the care and use of laboratory animals.

2.5. Experimental Design

Animals were randomly divided into seven groups (n = 6 per group):

- **Group I (Negative Control):** Received distilled water orally.
- **Group II (Positive Control):** Administered Imidacloprid (20 mg/kg/day, p.o.) for induction of infertility.
- **Group III (Test I):** Imidacloprid + *Vitex agnus-castus* extract (200 mg/kg/day, p.o.).

- **Group IV (Test II):** Imidacloprid + *Nigella sativa* extract (300 mg/kg/day, p.o.).
- **Group V (Test III):** Imidacloprid + combination of *Vitex agnus-castus* (200 mg/kg) and *Nigella sativa* (300 mg/kg).
- **Group VI (Standard):** Imidacloprid + Clomiphene citrate (1 mg/kg/day, p.o.).
- **Group VII (Male rats):** Untreated, used for mating with females at a ratio of 3:1.

Imidacloprid was administered for 28 consecutive days to induce infertility. Following induction, the respective treatments were given for another 28 days.

2.6. Induction of Infertility in female rats and treatment schedule

Infertility was experimentally induced in female Wistar albino rats by oral administration of Imidacloprid (10 mg/kg/day) for 28 consecutive days. Following induction, animals were given a two-day rest period before initiation of treatment. Group I (Normal control) animals received only standard pellet diet and water, while Group II (Positive control) animals were administered Imidacloprid but received no therapeutic intervention. Group III animals were treated with *Vitex agnus-castus* extract (250 mg/kg/day, p.o.) for 28 days, whereas Group IV animals received *Nigella sativa* extract (150 mg/kg/day, p.o.) for 28 days [16,17]. Group V animals were administered both *Vitex agnus-castus* (250 mg/kg/day, p.o.) and *Nigella sativa* (150 mg/kg/day, p.o.) in combination at a one-hour interval for 28 days to evaluate synergistic efficacy. Group VI animals were treated with the standard drug clomiphene citrate (1 mg/kg/day, p.o.) for 9 days as a reference therapeutic control.[18] All test substances and standard drug were freshly prepared prior to administration, and treatment schedules were designed in accordance with established reproductive pharmacology protocols.

2.7. Blood Collection and determination of Hormonal Levels

At the end of the experimental period, blood samples were collected from female rats via the retro-orbital venous plexus under light anaesthesia. Animals were anesthetized using inhalation anaesthesia (isoflurane, 3-5% for induction and 1.5-2.5% for maintenance). After confirming adequate anaesthetic depth, the rat was positioned in lateral recumbency, and the medial canthus of the eye was gently exposed. A heparinized microcapillary tube (75 mm) was inserted into the retro-orbital plexus at a slight downward and backward angle until blood filled the tube by capillary action. The desired volume of blood was collected, and sterile gauze was immediately applied with light pressure to minimize bleeding and hematoma formation. [19, 20] . The collected blood samples were transferred into plain

serum tubes and allowed to clot at room temperature. Serum was separated by centrifugation at 3000 rpm for 15 minutes and stored at −20 °C until further analysis. Serum concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen, and progesterone were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. The obtained values were used to assess alterations in reproductive endocrine function and to correlate hormonal imbalance with infertility in experimental groups.[21]

2.8. Pregnancy Confirmation and Pup Birth

Following hematological evaluation, female rats from each experimental group were cohabited with proven fertile male rats at a ratio of 3:1 in standard breeding cages. The gestation period in Wistar rats typically ranges from 21 to 23 days, during which fertilized embryos undergo rapid intrauterine development.[22] Pregnancy confirmation was based on successful delivery of pups, with litter size serving as an indicator of reproductive outcome. The number of pups born per litter was recorded for each group, and the fertility index was calculated to assess treatment efficacy. Comparative analysis of pup birth rates across experimental groups was used to evaluate the protective and therapeutic potential of *Vitex agnus-castus*, *Nigella sativa*, their combination, and standard treatment against Imidacloprid-induced infertility.[23]

2.9. Histopathological Studies of Ovarian and Uterine tissues

At the termination of the experiment, animals were anesthetized and sacrificed by cervical dislocation in accordance with the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines. The uterus and ovaries were excised through midline abdominal incision, carefully isolated, blotted, and weighed. The tissues were immediately fixed in 10% neutral buffered formalin for 24-48 hours to preserve morphology. Following fixation, samples were dehydrated in graded alcohol series, cleared in xylene, and embedded in paraffin blocks. Tissue sections of 7 μm thickness were obtained using a rotary microtome and mounted on clean glass slides. Sections were stained with haematoxylin and eosin (H&E) for general histoarchitecture, and subsequently examined under a light microscope for structural alterations, follicular development, uterine endometrial integrity, and pathological changes associated with Imidacloprid-induced infertility and the effects of therapeutic interventions [24, 25].

2.10. Statistical Analysis

All data obtained from the experiments were quantitative and expressed as mean ± standard deviation (SD). Statistical evaluation was performed using appropriate tests to determine the significance of differences among groups. The Mann-Whitney U test was applied for pairwise comparisons between two independent groups. One-way analysis of variance (ANOVA) was employed for parameters influenced by a single factor, such as biochemical and hormonal markers, followed by post hoc comparisons where applicable. Two-way ANOVA was used to analyse parameters influenced by two independent factors, such as body weight, in order to assess both main and interaction effects. A p-value < 0.05 was considered statistically significant.

3. RESULT

3.1. LC-MS Analytical Profile for Structural identification of *Vitex agnus castus*

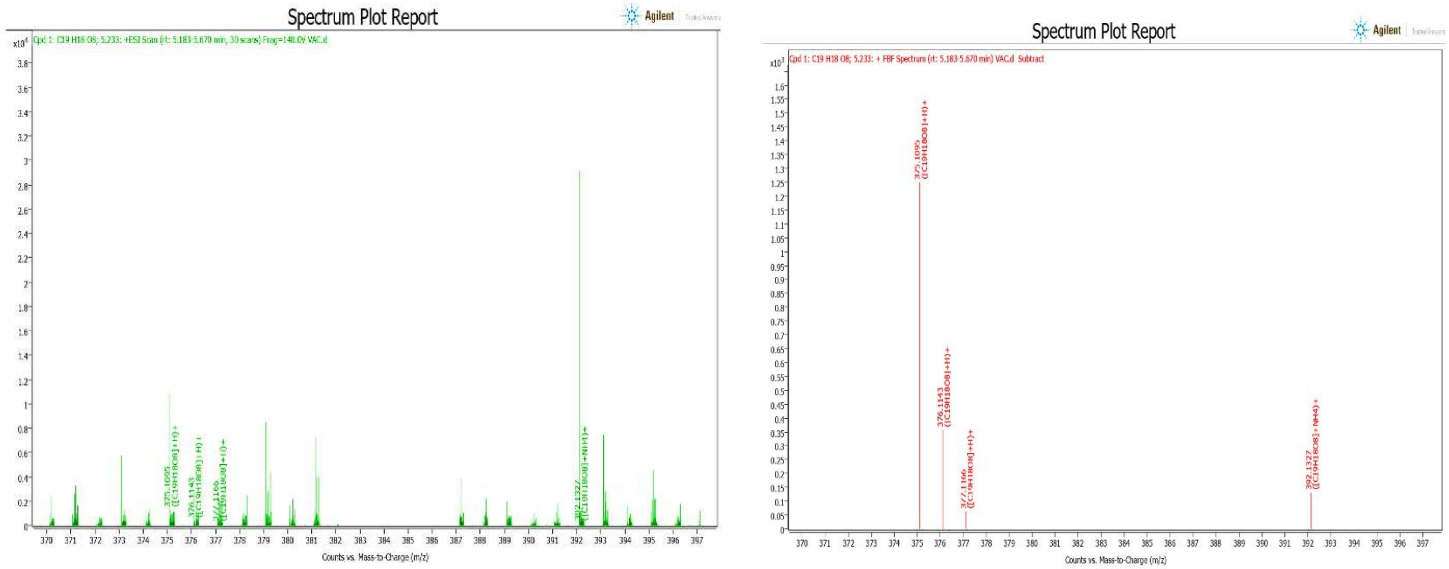


Fig.1: Diagrammatic graph representation of LC-MS/MS of phytoconstituent Casticin

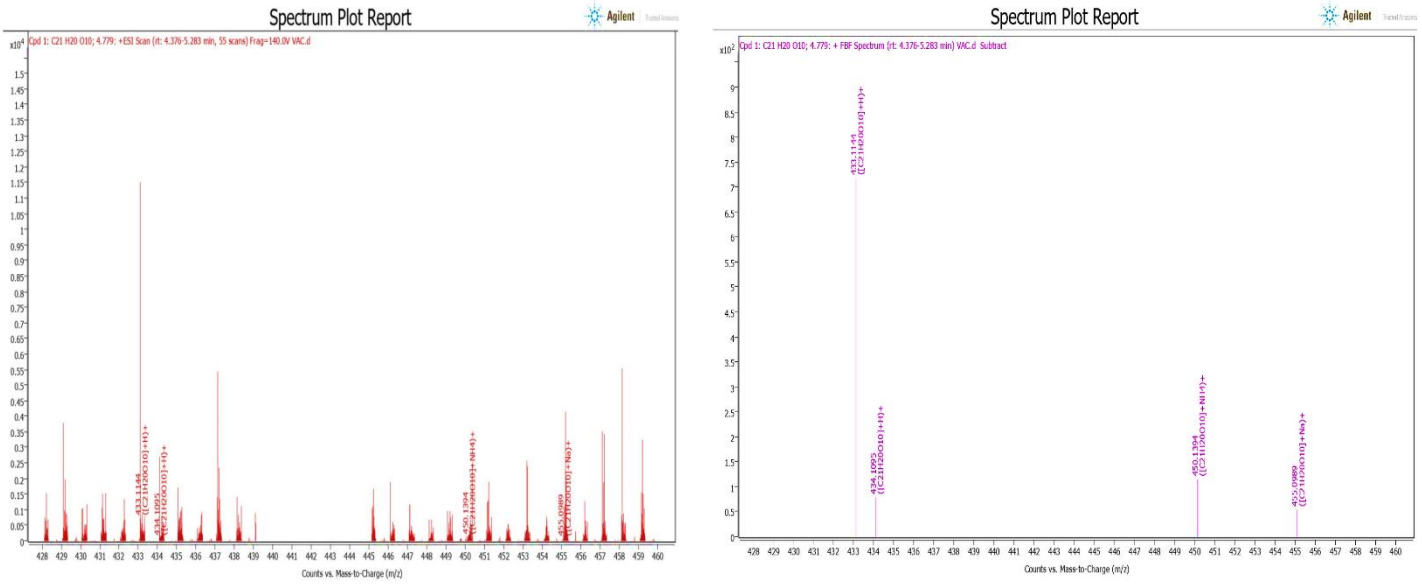


Fig.2 Diagrammatic graph representation ofLC-MS/MS of phytoconstituent Vitexin

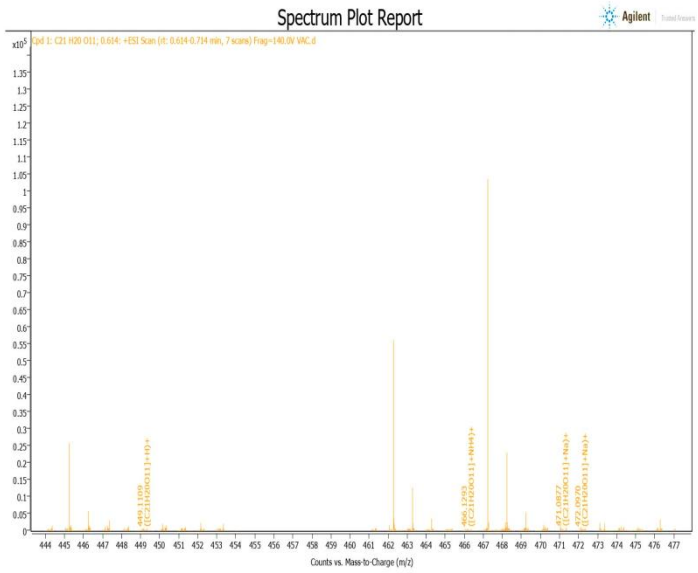
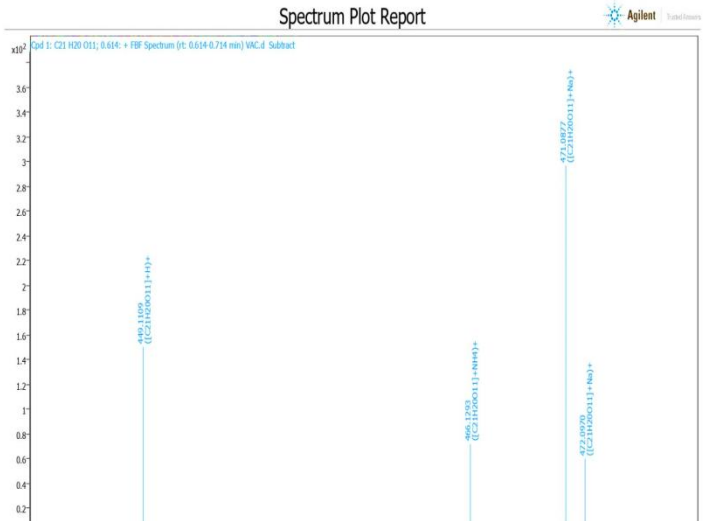
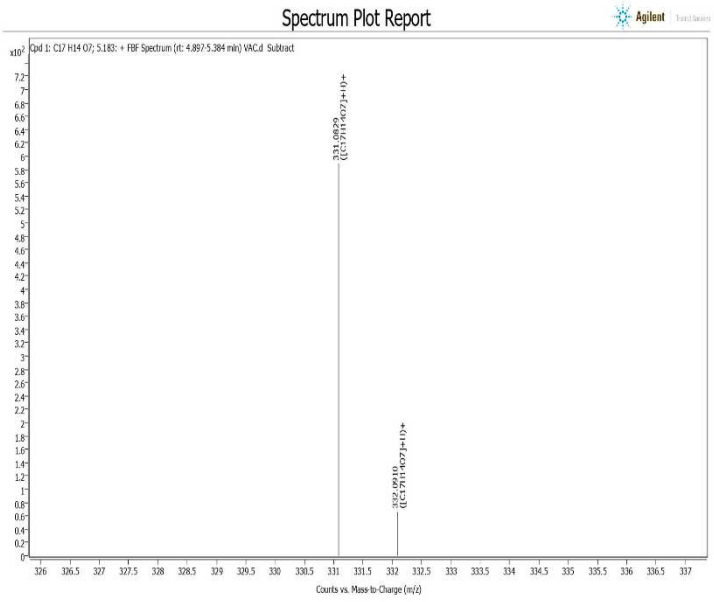


Fig.3: Diagrammatic graph representation ofLC-MS/MS of phytoconstituent Orientin





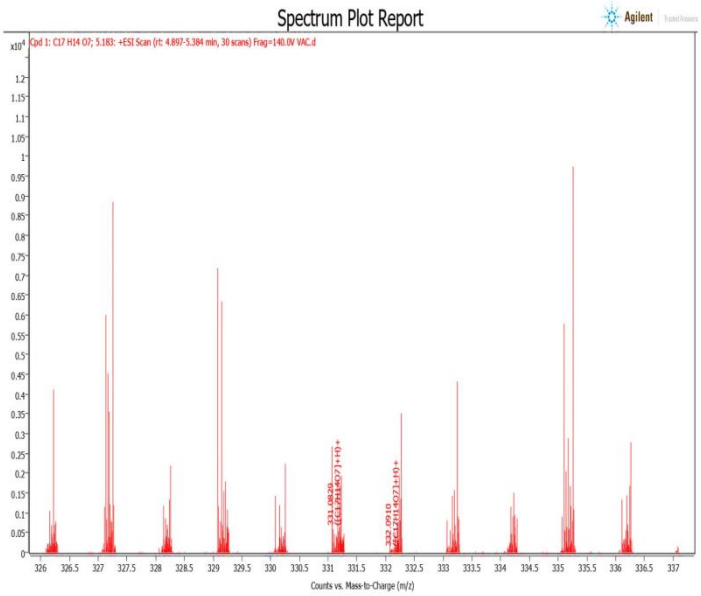


Fig.4: Diagrammatic graph representation of LC-MS/MS of phytoconstituent Penduletin

LC-MS Analytical Profile for Structural identification of *Nigella sativa*

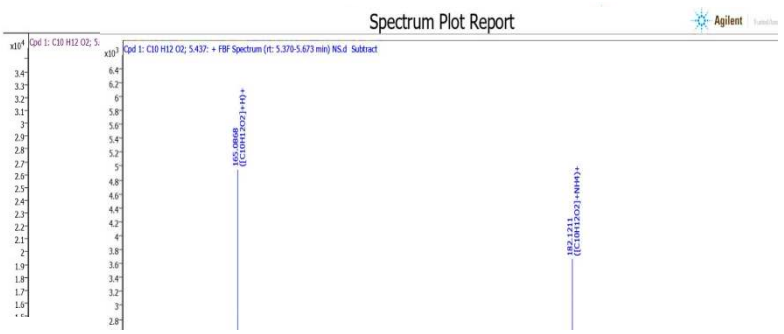


Fig.5: Diagrammatic graph representation ofLC-MS/MS of phytoconstituent Thymoquinone

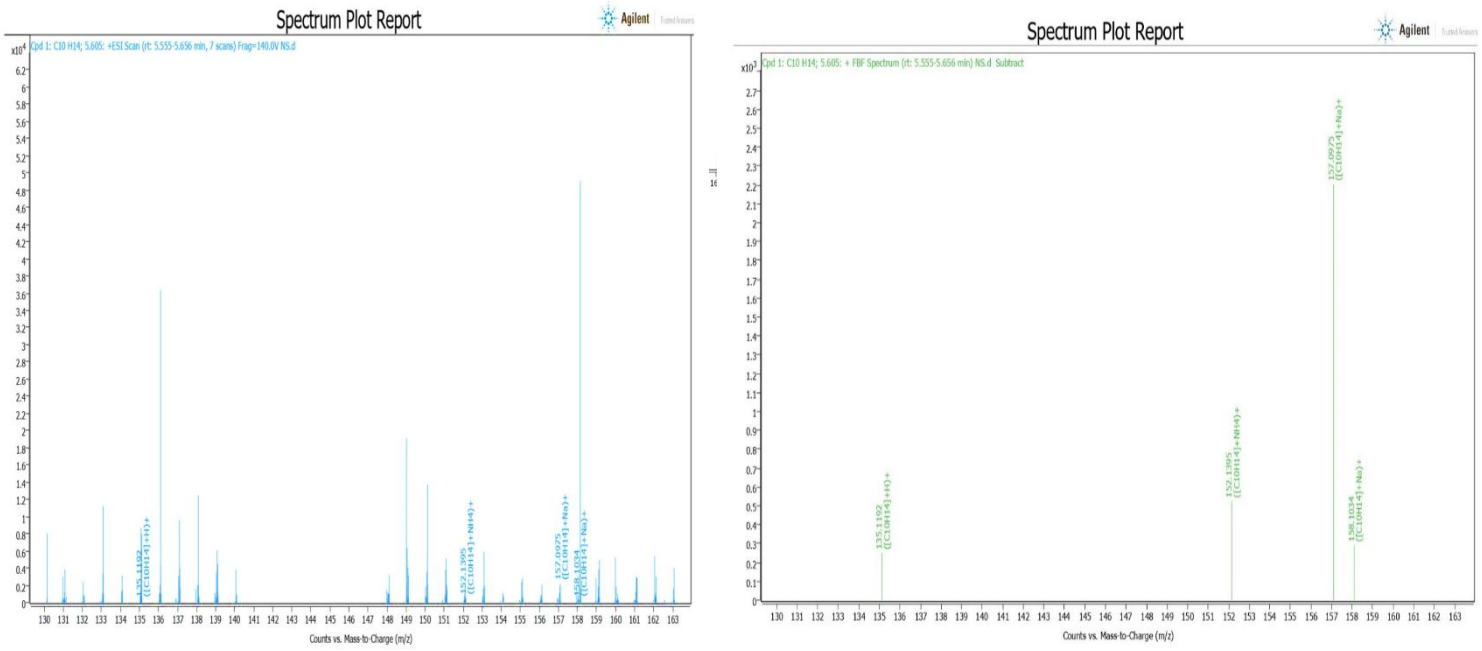


Fig.6: Diagrammatic graph representation ofLC-MS/MS of phytoconstituent p-cymene

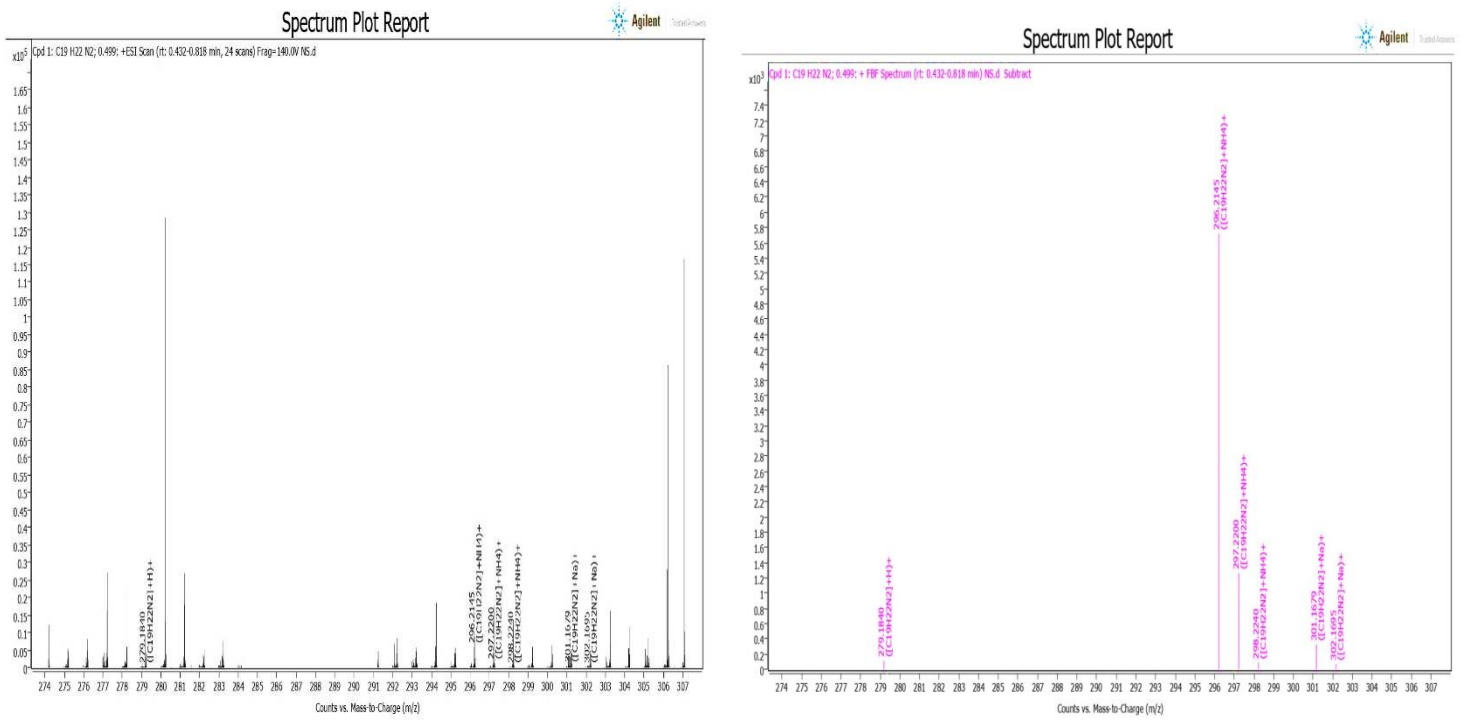


Fig.7: Diagrammatic graph representation ofLC-MS/MS of phytoconstituent Nigellidine

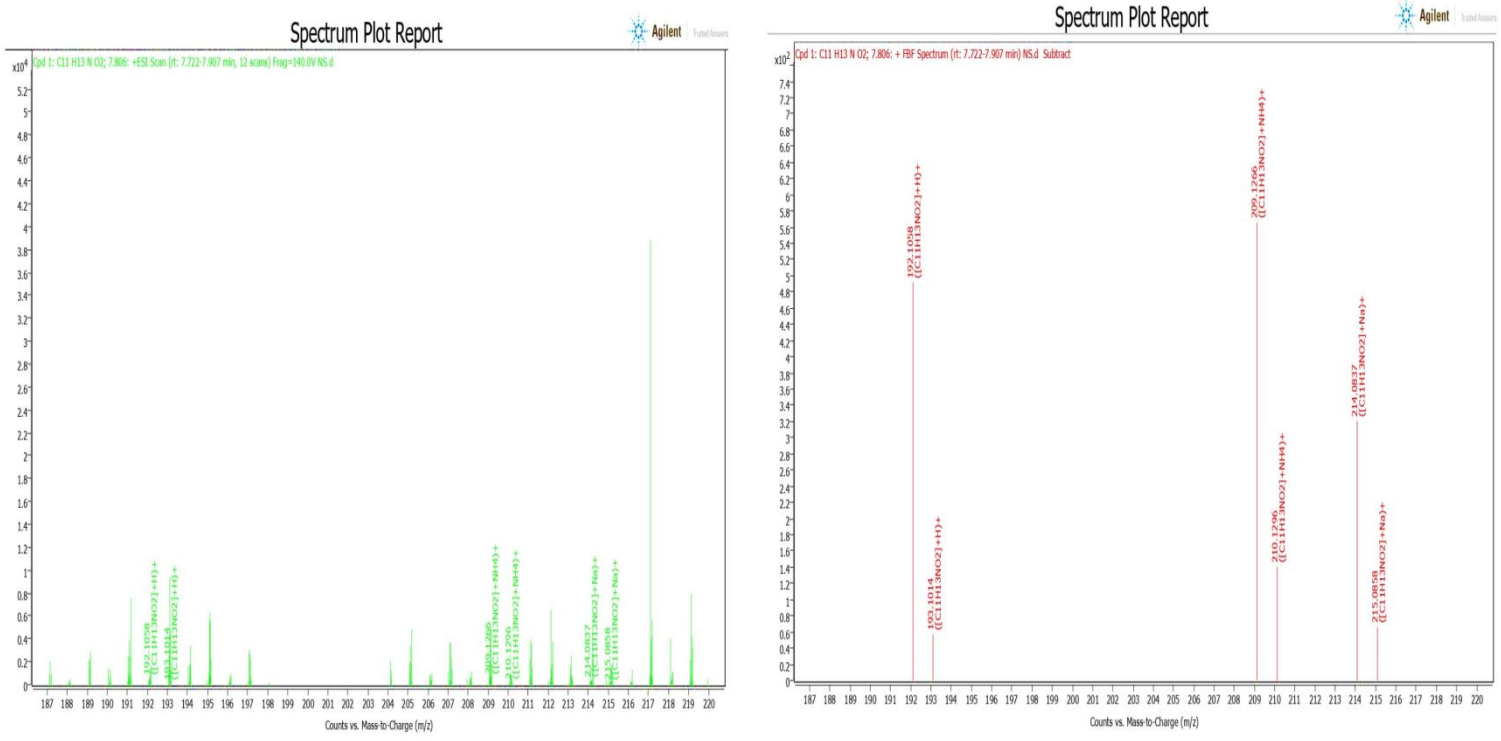


Fig.8: Diagrammatic graph representation ofLC-MS/MS of phytoconstituent Nigellicine

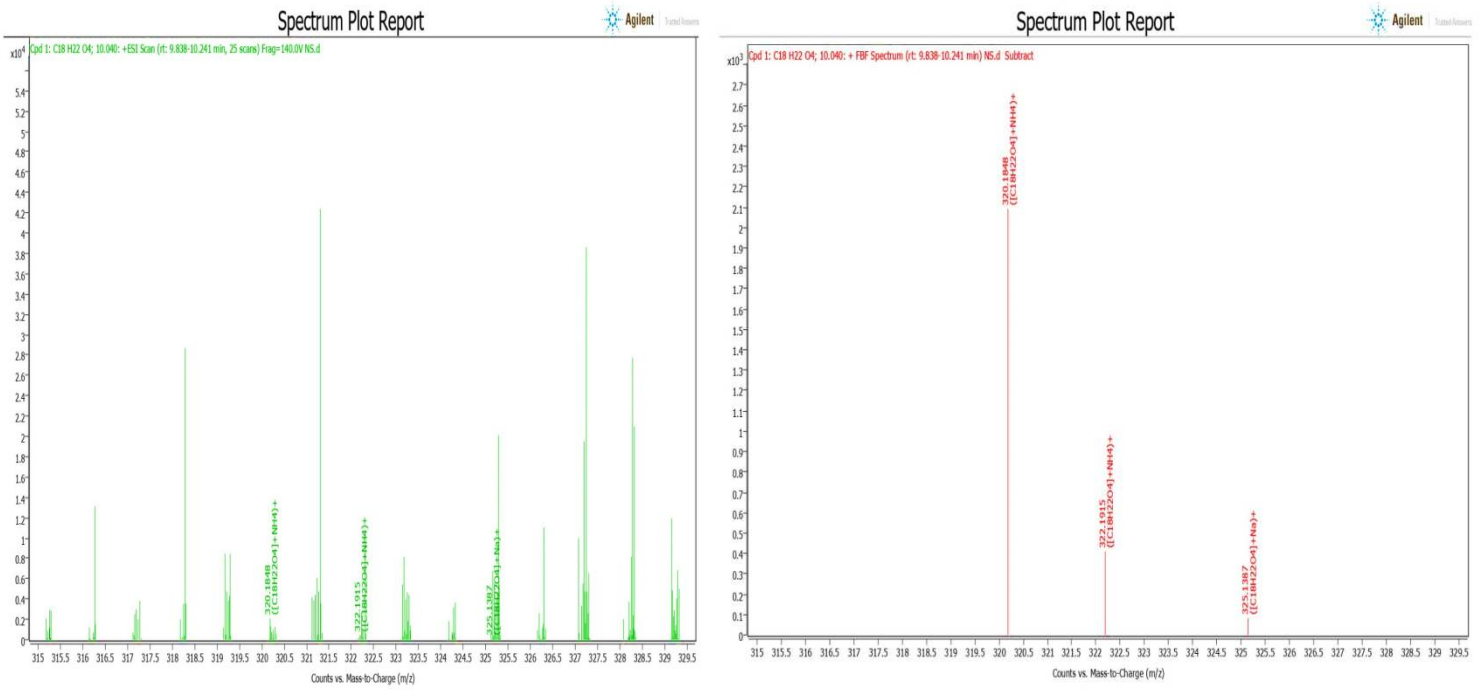


Fig.9: Diagrammatic graph representation ofLC-MS/MS of phytoconstituentNigellone

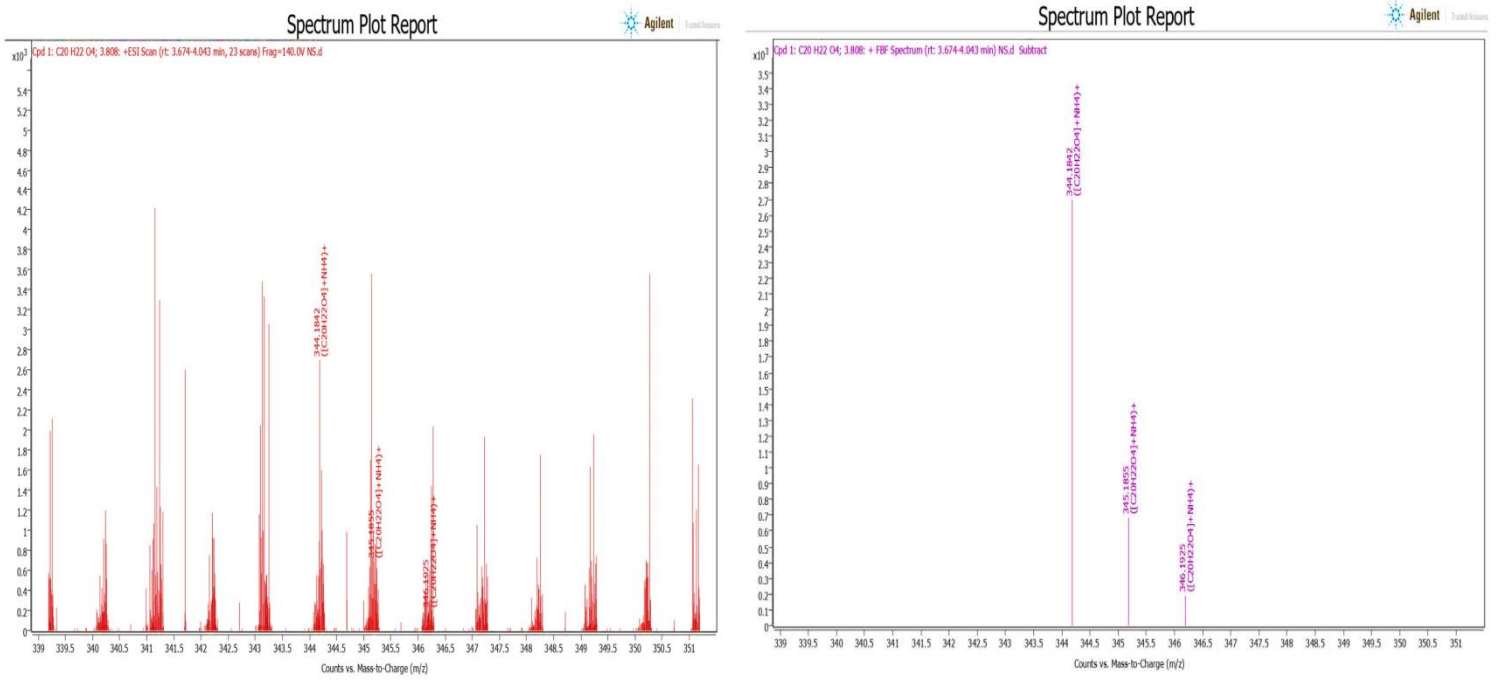


Fig.10: Diagrammatic graph representation ofLC-MS/MS of phytoconstituentDithymoquinone

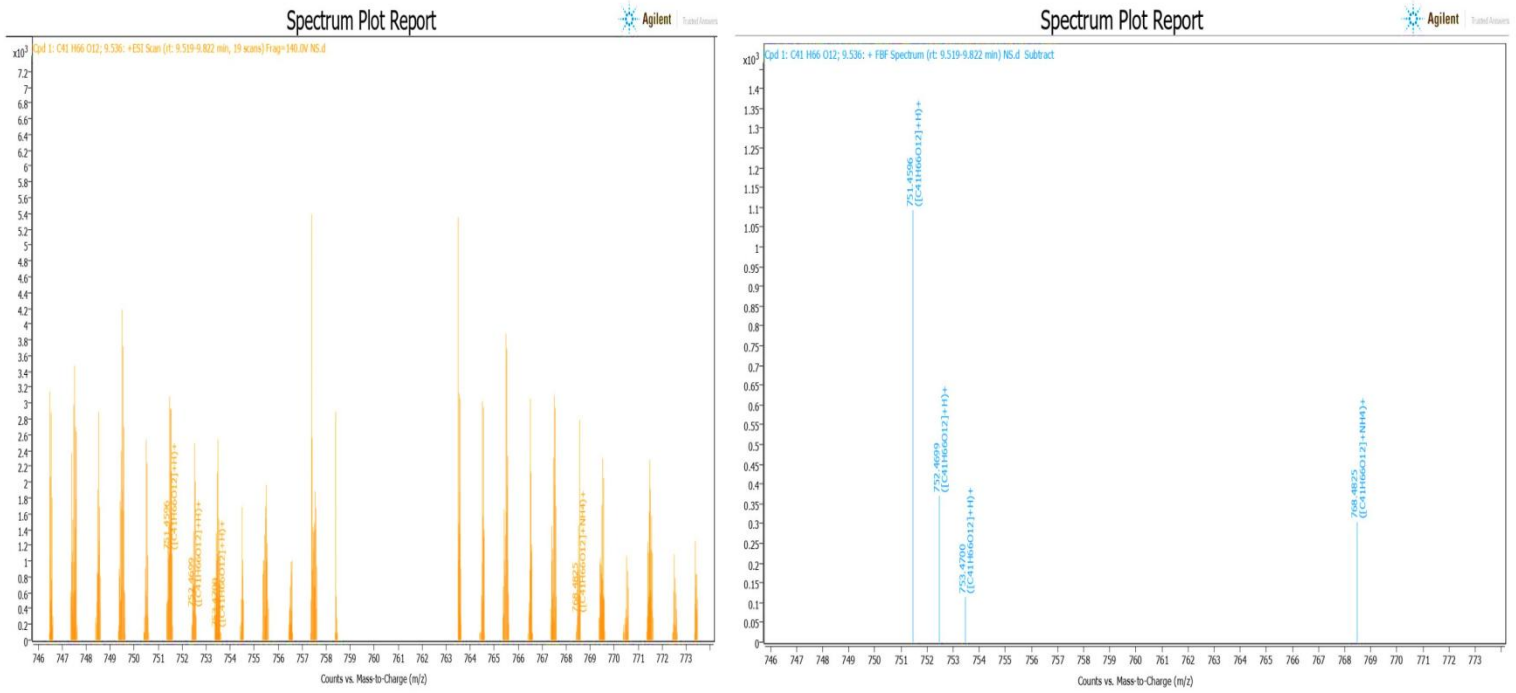


Fig.11: Diagrammatic graph representation ofLC-MS/MS of phytoconstituentα-Hederin

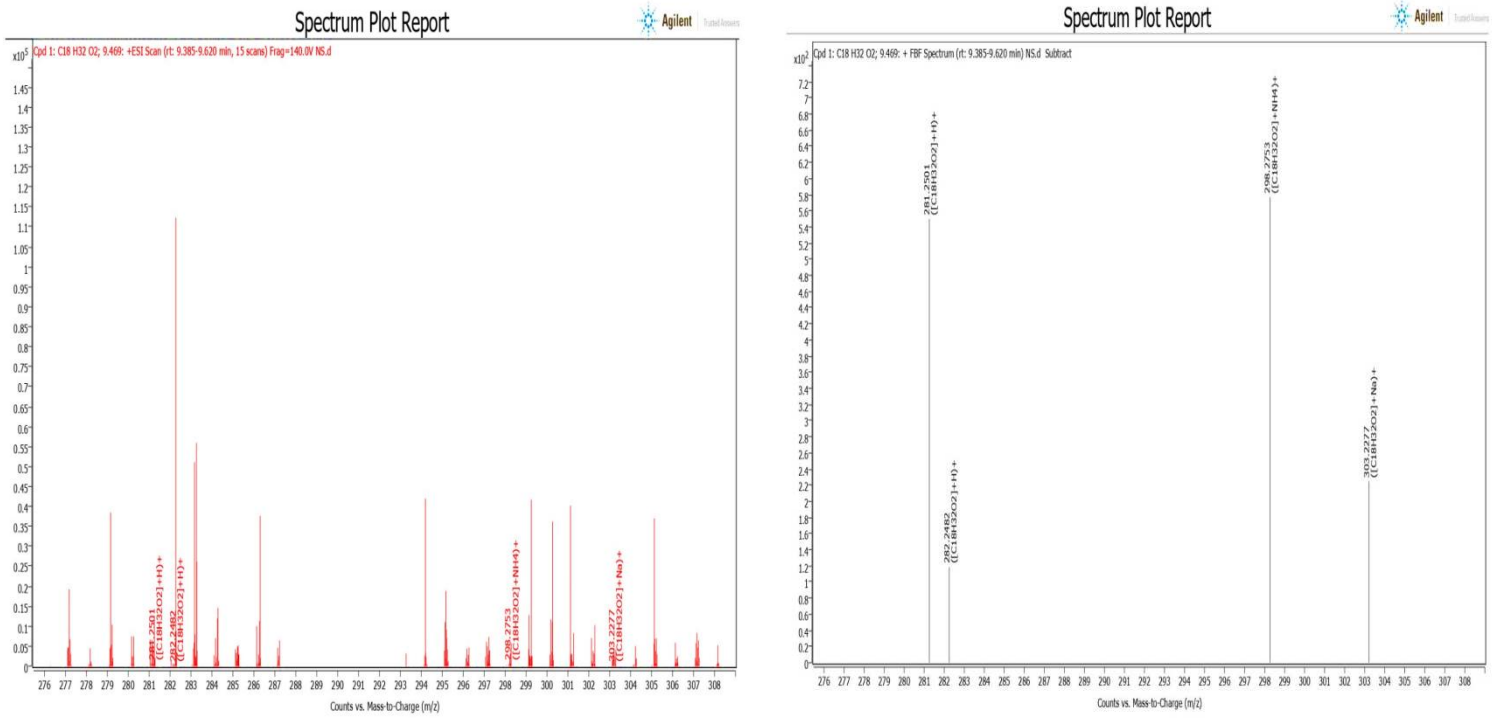


Fig.12: Diagrammatic graph representation ofLC-MS/MS of phytoconstituent Linoleic acid

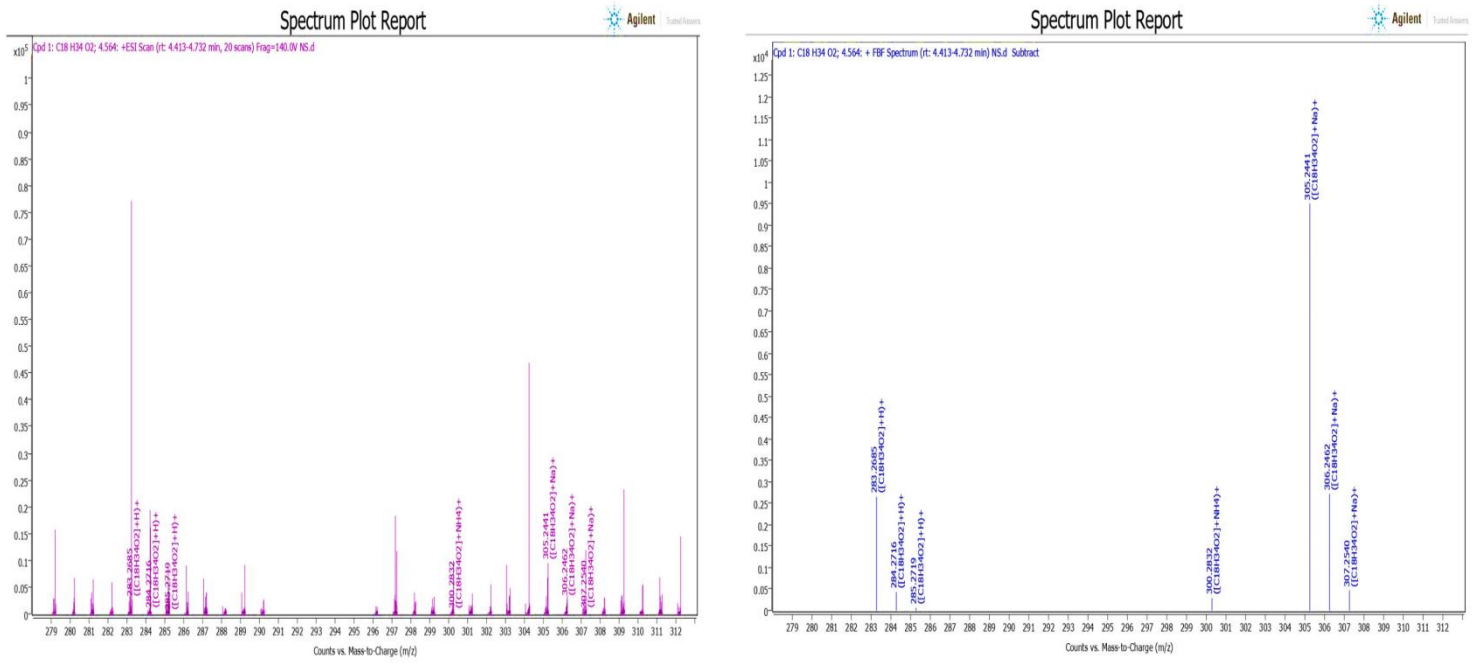


Fig.13: Diagrammatic graph representation of LC-MS/MS of phytoconstituentOleic acid

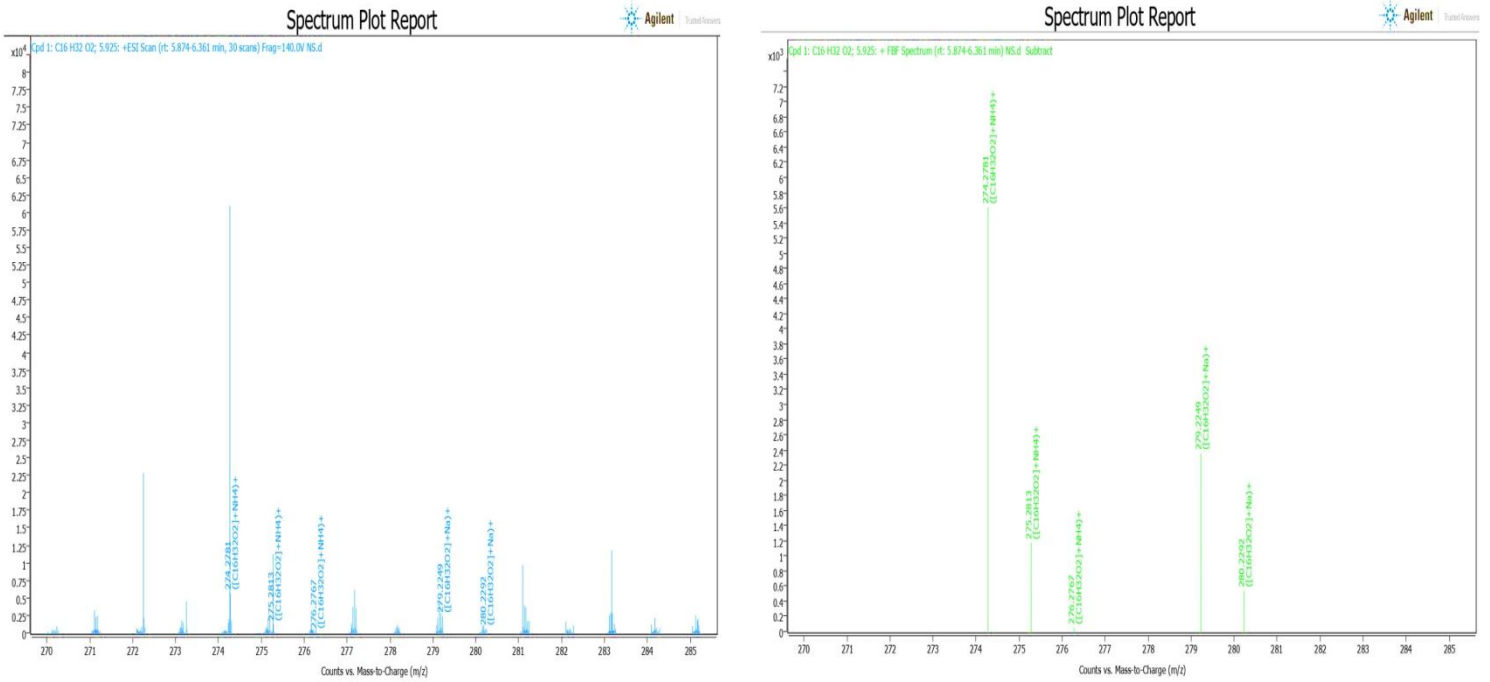
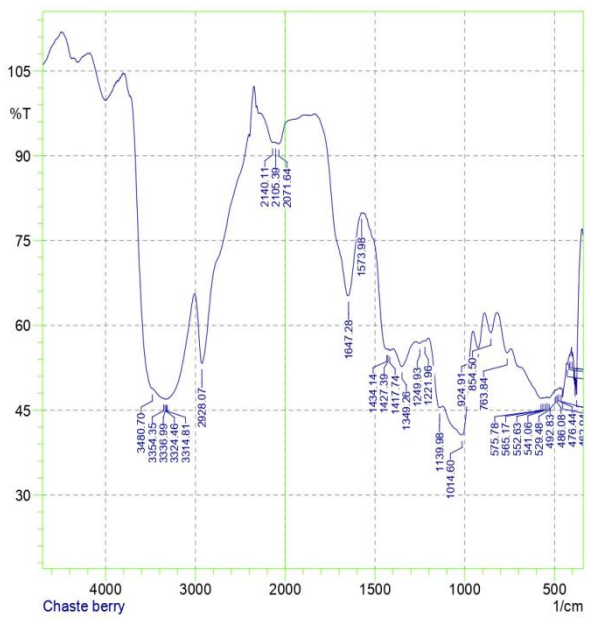
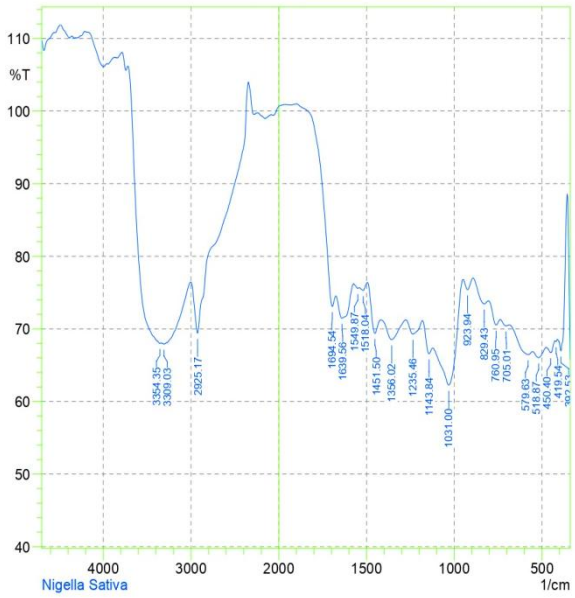


Fig.14: Diagrammatic graph representation ofLC-MS/MS of phytoconstituent Palmitic acid

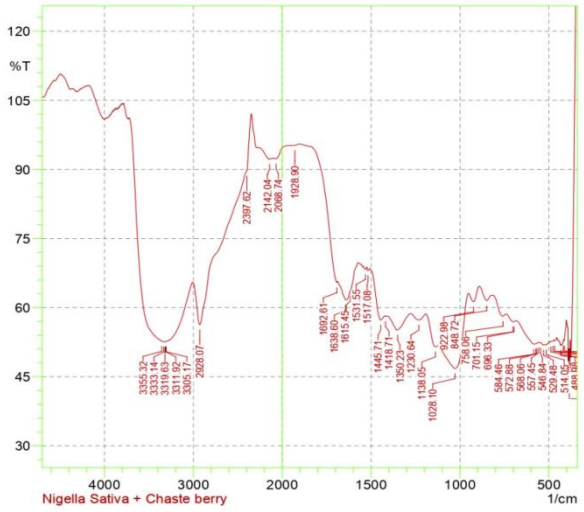
3.3. FTIR Spectral Characterization and Functional group Identification of *Vitex agnus castus* and *Nigella sativa*



(a)



(b)



(c)

Fig.15:(a)Diagrammatic graph representation of FTIR of *Vitex agnus castus*(b)Diagrammatic graph representation of FTIR of *Nigella sativa* (c) Diagrammatic graph representation of FTIR of *Vitex agnus castus* and *Nigella sativa L.*

3.4. *In-Vivo* Pharmacological evaluation of *Vitex agnus castus* and *Nigella sativa L* on different parameters.

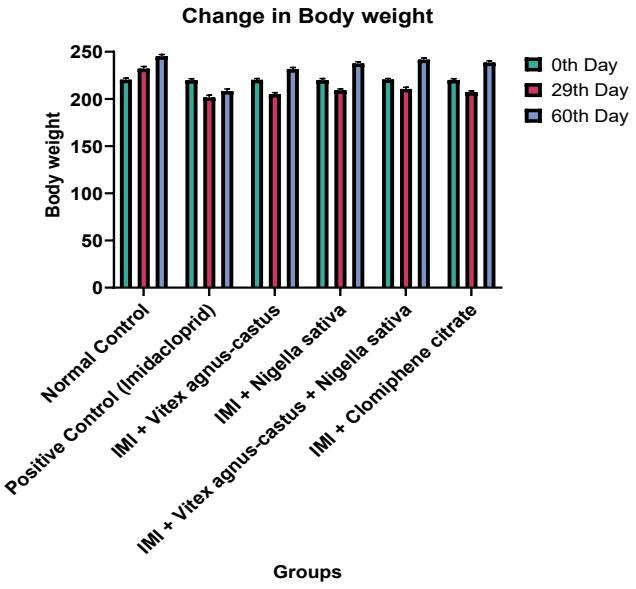


Fig.16: Diagrammatic graph representation of Impact of Therapeutic Interventions on Body Weight Changes in Rats

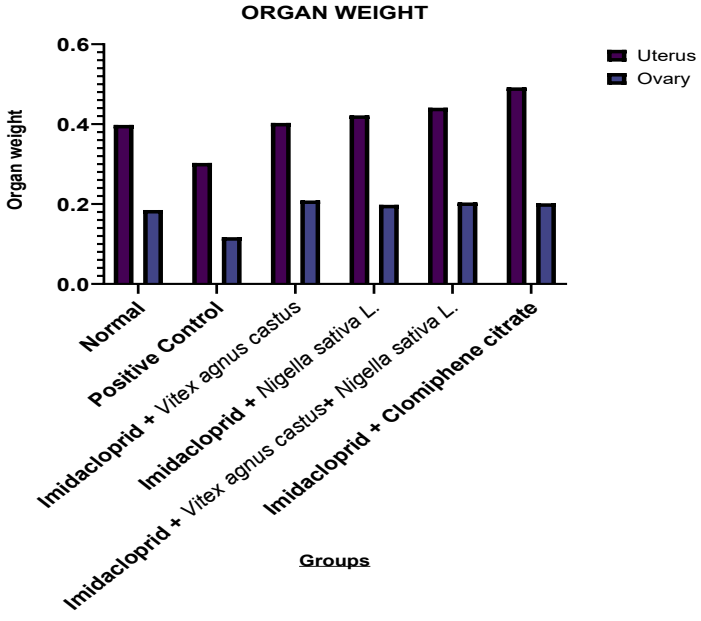


Fig.17: Diagrammatic graph representation of Effect of Treatments on Isolated Organ Weights in Experimental Groups

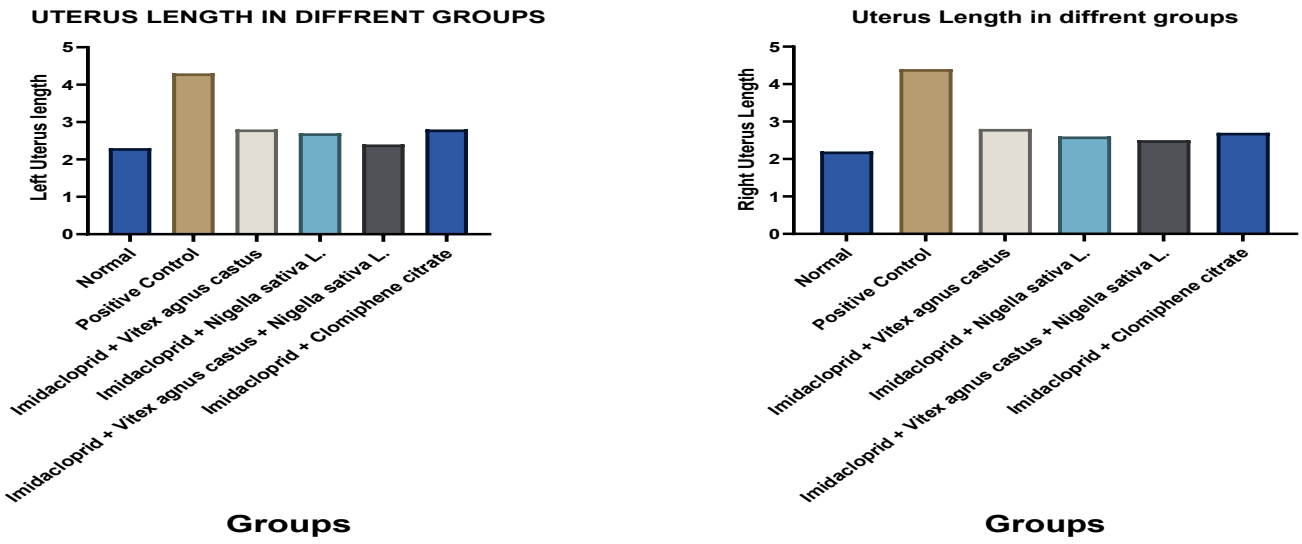


Fig.18: Diagrammatic graphical representation of Effect of Treatments on Bilateral Uterine Length in Experimental Groups

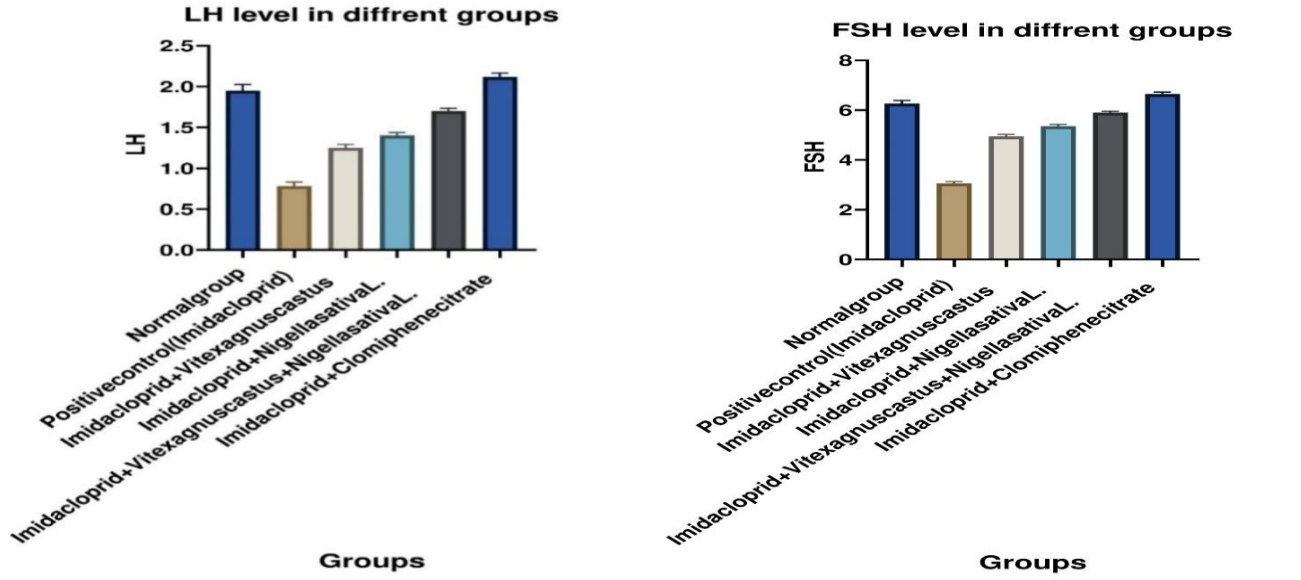


Fig.19: Graphical representation of Effect of Treatments on Gonadotropin (LH and FSH) Levels inExperimental Groups

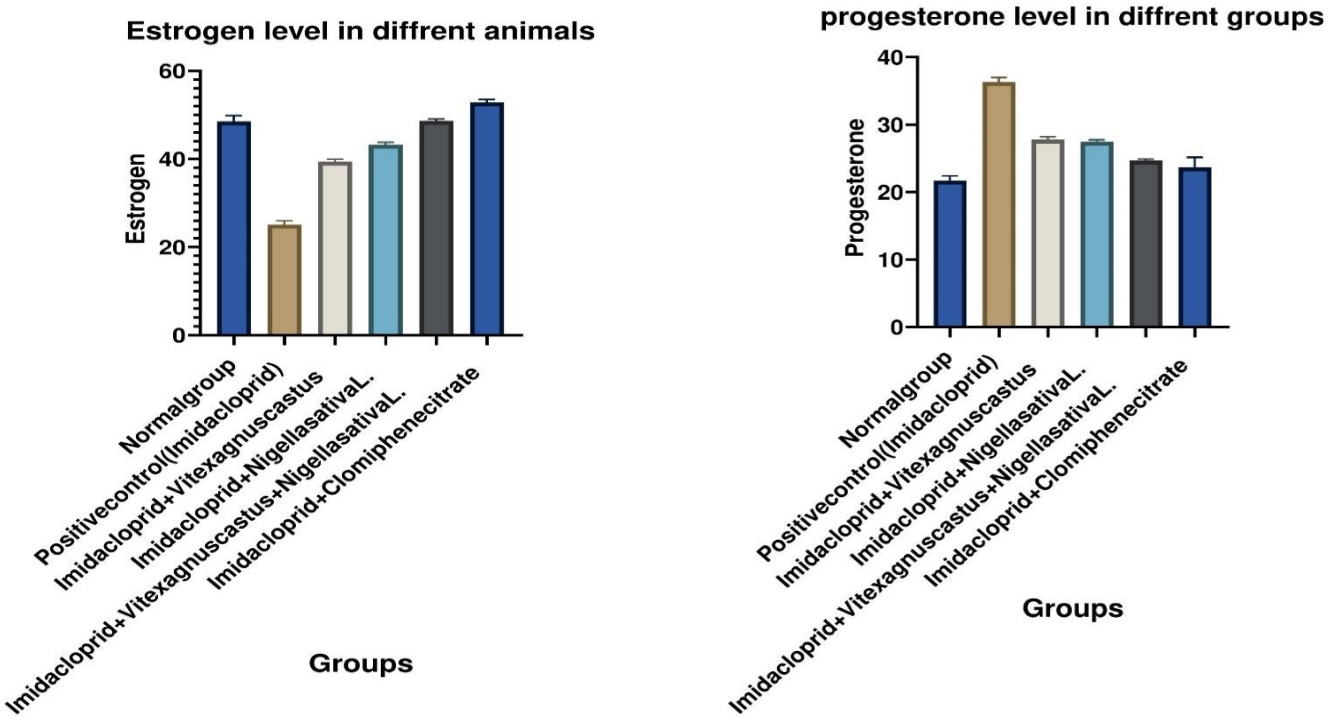


Fig.20: Graphical representation of Effect of Treatments on Estrogen and Progesterone Levels in Experimental Groups

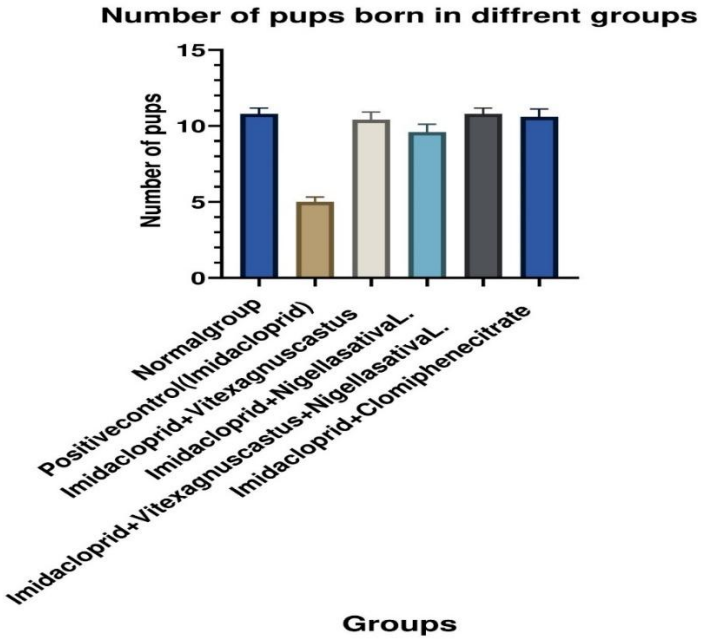
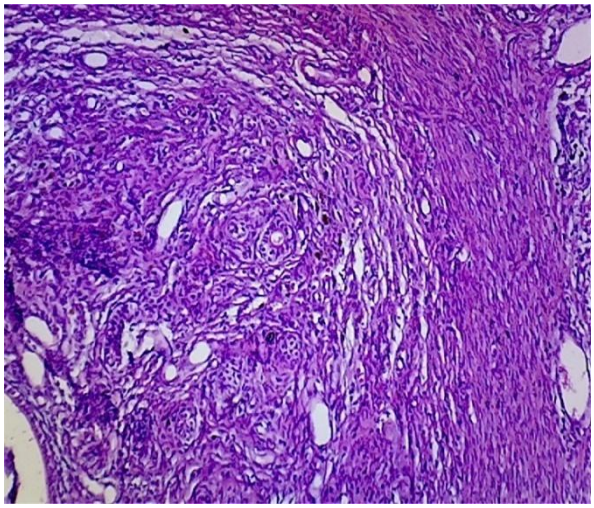
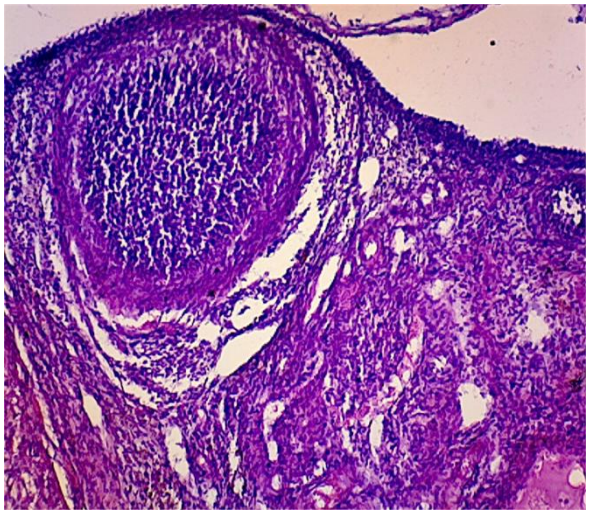


Fig.21: Graphical representation of Effect of Treatments on Litter Size Across Experimental Groups

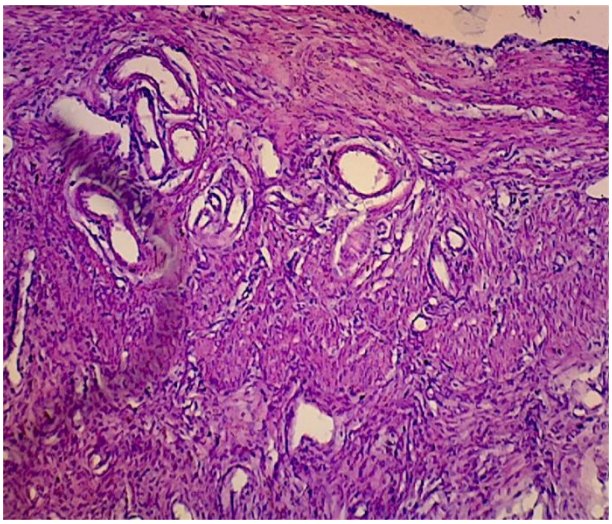


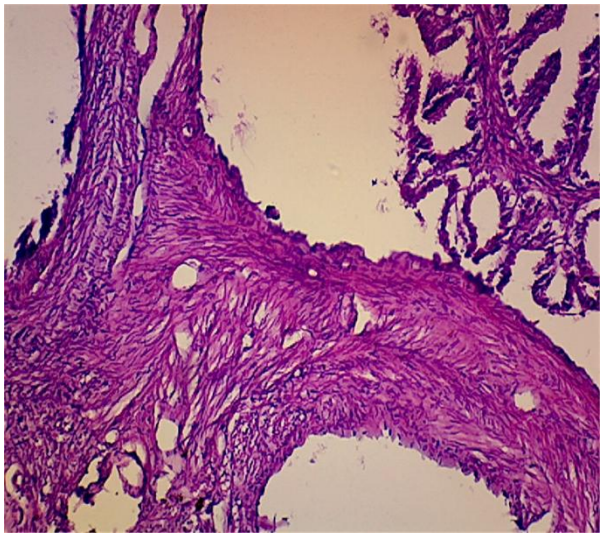


(a) **(b)**

Fig.22: (a) Histopathology of Uterus (Normal group): Normal endometrium with intact glands and epithelial lining

(b) Histopathology of Ovary (Normal group): Healthy follicles and corpus luteum with no degeneration

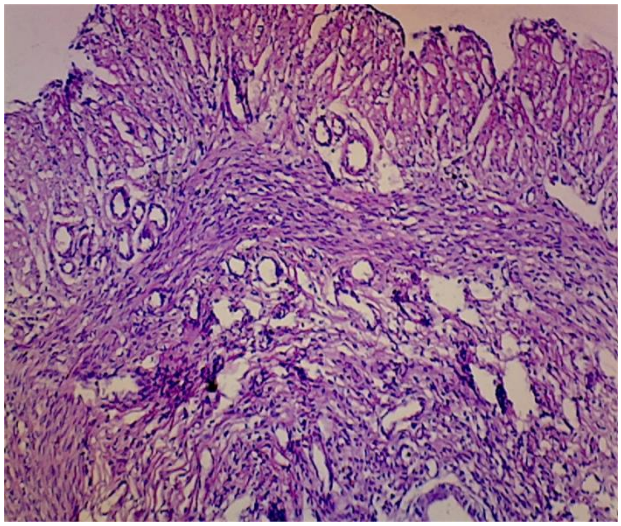




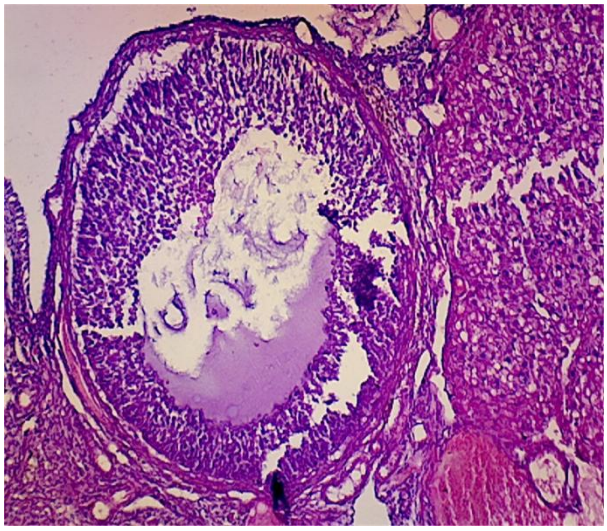
(a) **(b)**

Fig.23: (a) Histopathology of Uterus (Positive control group):Thinned endometrium with glandular degeneration and inflammatory infiltration

(b) Histopathology of Ovary (Positive control group):Extensive follicular atresia and degenerated oocytes with absent corpus luteum



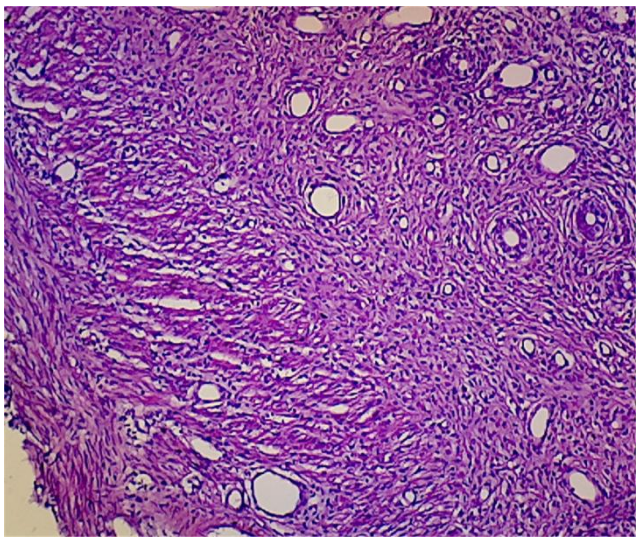
(a)



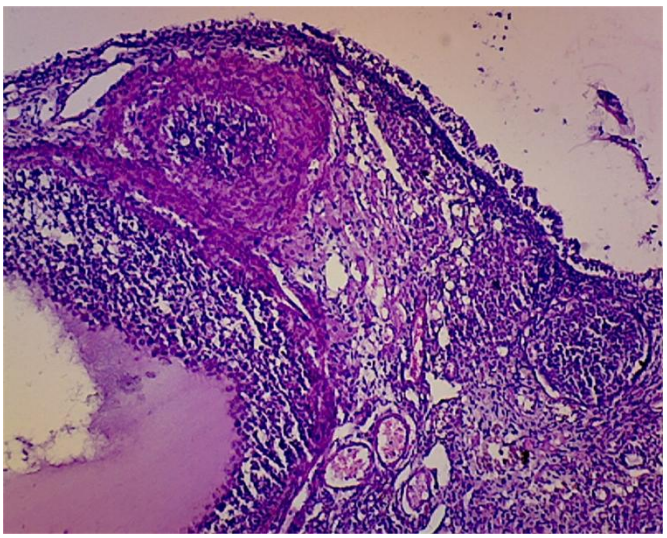
(b)

Fig.24: (a) Histopathology of Uterus Test group-I (Imidacloprid + *Vitex agnus castus*): Mild endometrial regeneration with partial restoration of glands

(b) Histopathology of Ovary Test group-I (Imidacloprid + *Vitex agnus castus*):Moderate follicular development and occasional corpus luteum



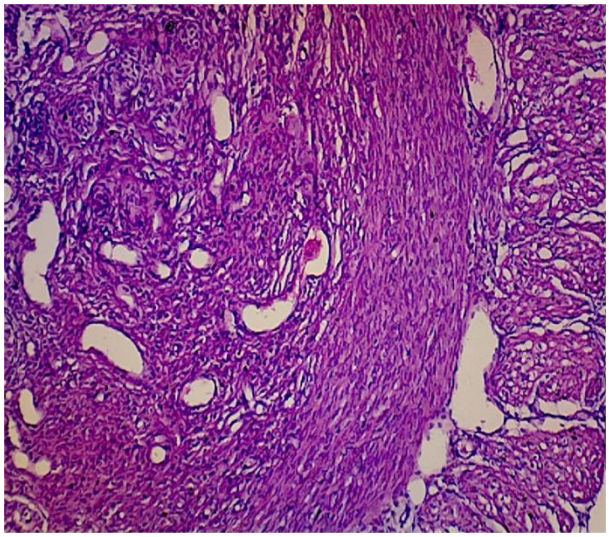
(a)



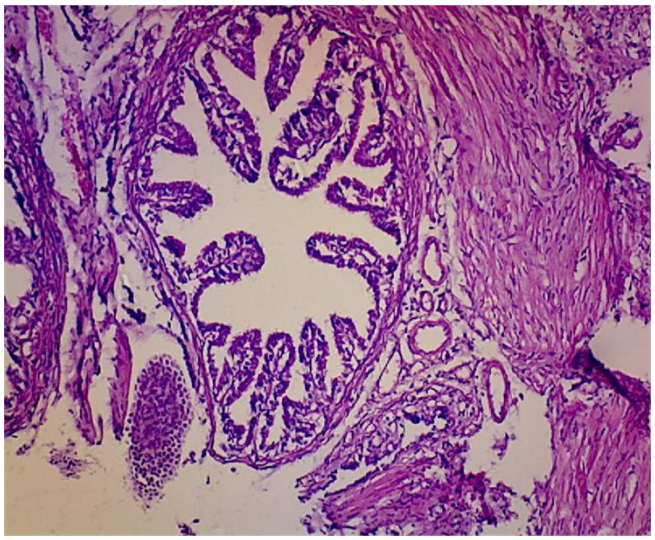
(b)

Fig.25: (a) Histopathology of Uterus Test group-II (Imidacloprid + *Nigella sativa L.*):Improved glandular structure with reduced congestion and mild epithelial recovery

(b) Histopathology of Ovary Test group-II (Imidacloprid + *Nigella sativa L.*):Active folliculogenesis with restored corpus luteum and reduced atresia



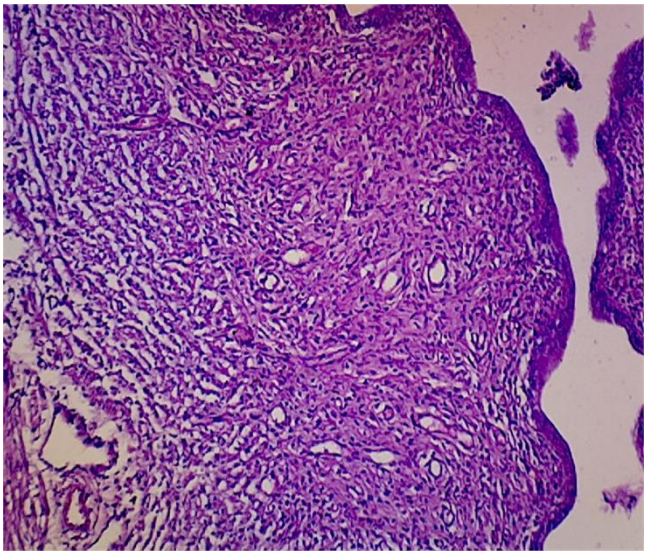
(a)



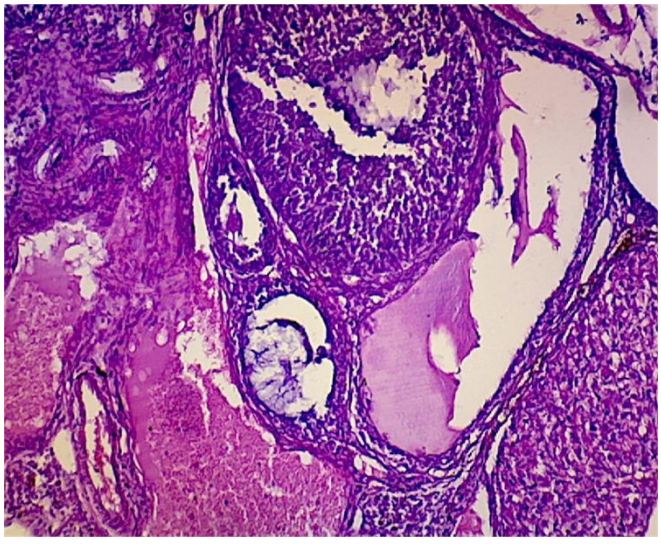
(b)

Fig.26: (a) Histopathology of Uterus Test group-III (Imidacloprid + *Vitex agnus castus* + *Nigella sativa L.*): Marked endometrial repair with well-organized glands and stroma.

(b) Histopathology of Ovary Test group-III (Imidacloprid + *Vitex agnus castus* + *Nigella sativa L.*):Well-developed follicles and corpus luteum with minimal signs ofdegeneration



(a)



(b)

Fig.27: (a) Histopathology of Uterus standard group (Imidacloprid + Clomiphene citrate): Almost complete normalization of endometrial architecture, resembling control

(b) Histopathology of Ovary standard group (Imidacloprid + Clomiphene citrate):Near-normal ovarian structure with multiple mature follicles and active corpus luteum

4. DISCUSSION AND CONCLUSION

The current study comprehensively demonstrates that phytotherapeutic intervention with *Vitex agnus-castus* and *Nigella sativa*, individually and in combination, exerts pronounced protective effects against imidacloprid-induced reproductive toxicity in female rats, with efficacy comparable to the synthetic ovulation-inducing agent clomiphene citrate. Phytochemical profiling by LC-MS unequivocally confirmed the presence of structurally diverse bioactive compounds: in *Vitex agnus-castus*, major peaks at m/z 375.12 (casticin), 431.10 (vitexin), 471.09 (orientin), and 311.09 (penduletin) validated the dominance of flavonoid glycosides and lignans with estrogenic and antioxidant properties, while in *Nigella sativa*, abundant peaks at m/z 165.09 (thymoquinone), 157.07 (p-cymene sodium adduct), 295.21 (nigellidine), 209.11 (nigellicine), 309.13 (nigellone), 345.13 (dithymoquinone), 751.49 (α-hederin), 285.27 (linoleic acid), 305.26 (oleic acid), and 273.25 (palmitic acid) confirmed the presence of terpenoids, quinones, alkaloids, and fatty acids with well-documented antioxidant, anti-inflammatory, and endocrine-modulating activities. These findings were corroborated by FTIR analysis, which revealed intense O–H stretching bands at ~3348 cm^{-1} and 3365 cm^{-1}, C=O absorptions at 1692–1694 cm^{-1}, and C–H stretching at ~2924 cm^{-1} in both extracts, indicating abundant hydroxyl, carbonyl, and aliphatic functional groups, while additional C≡N/C≡C bands at ~2100 cm^{-1} and strong C–O stretching at 1240–1139 cm^{-1} confirmed the presence of alkynes/nitriles and ester/ether moieties. Collectively, these phytoconstituents represent a chemically diverse arsenal capable of targeting multiple pathways of toxicant-induced reproductive dysfunction. Functionally, exposure to imidacloprid (Positive Control group) induced significant systemic and reproductive toxicity, as evidenced by a marked reduction in body weight (from 183.6 ± 4.2 g at baseline to 142.8 ± 3.7 g at day 60, $p < 0.01$ vs. Normal Control) compared to stable weight gain in the Normal Control group (187.4 ± 5.1 g to 202.7 ± 4.8 g). Similarly, ovarian and uterine weights were significantly reduced (ovary: 25.3 ± 1.2 mg vs. 39.6 ± 1.8 mg in controls; uterus: 108.2 ± 4.7 mg vs. 164.3 ± 6.1 mg; $p < 0.01$), reflecting severe reproductive organ atrophy. Uterine length was paradoxically increased (left: 20.4 ± 0.6 mm vs. 14.3 ± 0.5 mm; right: 21.1 ± 0.7 mm vs. 14.6 ± 0.6 mm), suggestive of hypertrophy and inflammatory swelling. Endocrine disruption was profound, with LH and FSH levels declining by ~50% (LH: 2.1 ± 0.3 mIU/mL vs. 4.5 ± 0.4 mIU/mL; FSH: 1.8 ± 0.2 mIU/mL vs. 3.7 ± 0.3 mIU/mL in controls), accompanied by a sharp decrease in serum estrogen (18.6 ± 2.4 pg/mL vs. 44.7 ± 3.8 pg/mL; $p < 0.001$) and a paradoxical rise in progesterone (11.3 ± 1.7 ng/mL vs. 6.1 ± 1.1 ng/mL), indicative of impaired steroidogenesis and luteal phase

dysregulation. Fertility was severely compromised, as reflected in reduced litter size (3.1 ± 0.9 pups vs. 9.2 ± 1.3 in controls, $p < 0.001$). Histopathologically, the uterus exhibited endometrial thinning, glandular degeneration, and dense leukocytic infiltration, while ovarian sections revealed extensive follicular atresia, degenerated oocytes, and absence of corpus luteum, underscoring a collapse of folliculogenesis and ovulation. Remarkably, co-treatment with *Vitex agnus-castus* mitigated many of these adverse effects: body weight improved to 171.3 ± 4.2 g, ovarian weight increased to 31.7 ± 1.6 mg, uterine weight rose to 138.4 ± 5.2 mg, and gonadotropins partially normalized (LH: 3.4 ± 0.3 mIU/mL; FSH: 2.9 ± 0.2 mIU/mL). Estrogen levels improved to 33.1 ± 2.7 pg/mL, while progesterone decreased to 8.2 ± 1.3 ng/mL, consistent with partial restoration of ovarian steroidogenesis. Histological evaluation confirmed mild endometrial regeneration and partial restoration of corpus luteum, indicating resumed ovulatory activity. Similarly, *Nigella sativa* co-treatment yielded comparable protective effects, with body weight improving to 174.6 ± 3.9 g, ovarian weight 33.9 ± 1.5 mg, uterine weight 142.1 ± 4.8 mg, LH 3.6 ± 0.2 mIU/mL, FSH 3.1 ± 0.3 mIU/mL, estrogen 34.7 ± 3.1 pg/mL, and progesterone 7.9 ± 1.1 ng/mL; histologically, marked improvement was evident with active folliculogenesis and restoration of corpus luteum. Strikingly, the combined *Vitex agnus-castus* + *Nigella sativa* group displayed synergistic efficacy, with body weight approaching normal (186.8 ± 4.1 g), ovarian and uterine weights restored to near-control levels (38.2 ± 1.7 mg and 159.6 ± 6.3 mg, respectively), uterine length normalized (left: 15.2 ± 0.6 mm; right: 15.1 ± 0.5 mm), gonadotropins fully restored (LH: 4.3 ± 0.3 mIU/mL; FSH: 3.6 ± 0.2 mIU/mL), estrogen levels reaching 42.9 ± 3.4 pg/mL, and progesterone corrected to 6.8 ± 1.0 ng/mL. Fertility outcomes were particularly compelling, with litter size averaging 8.7 ± 1.2 pups, statistically indistinguishable from Normal Control and Clomiphene groups (9.1 ± 1.1 pups). Histopathology confirmed substantial uterine and ovarian recovery, characterized by well-organized endometrial glands, dense stromal architecture, multiple mature follicles, and an intact corpus luteum, closely mirroring the restorative effects seen with clomiphene citrate. Mechanistically, these protective actions can be explained by the complementary pharmacological activities of the two botanicals: *Vitex agnus-castus*constituents such as casticin and vitexin likely act through dopaminergic modulation of prolactin and phytoestrogenic agonism at estrogen receptors, thereby stimulating gonadotropin release and promoting follicular development, while *Nigella sativa*bioactives such as thymoquinone, nigellone, and linoleic acid provide robust antioxidant defense, attenuate lipid peroxidation, stabilize mitochondrial membranes, and inhibit inflammatory signaling, thereby preventing apoptosis of ovarian cells and preserving steroidogenic enzyme activity. The observed

synergism suggests that while *Vitex agnus-castus* primarily corrects central endocrine signaling via the HPG axis, *Nigella sativa* predominantly mitigates peripheral ovarian oxidative stress, together restoring both systemic and local determinants of fertility. Importantly, the magnitude of recovery in the combined herbal group not only paralleled but in certain parameters (e.g., normalized progesterone, histoarchitecture) exceeded clomiphene citrate, highlighting its translational potential as a safer phytotherapeutic alternative with fewer adverse effects. These findings are consistent with earlier reports on the fertility-enhancing effects of each extract but provide the first experimental evidence for their synergistic co-administration in pesticide-induced reproductive toxicity. From a translational perspective, the results underscore the growing importance of polyherbal formulations as rationally designed multi-target therapeutics capable of addressing complex endocrine toxicities where single-drug interventions may fail. However, limitations include the absence of oxidative stress biomarkers (e.g., MDA, SOD, CAT), apoptosis markers (e.g., caspase-3, Bax/Bcl-2), and receptor-binding assays, which could further elucidate mechanistic pathways; future work should integrate such molecular profiling with pharmacokinetic assessments to determine bioavailability and tissue distribution of active constituents, alongside long-term reproductive outcomes across multiple estrous cycles. In conclusion, this study establishes that *Vitex agnus-castus* and *Nigella sativa* possess substantial protective and restorative potential against imidacloprid-induced reproductive toxicity, achieved via phytoconstituent-driven normalization of gonadotropins, estrogen–progesterone balance, reproductive organ morphology, and fertility outcomes, with their combination exerting synergistic effects that rival the efficacy of clomiphene citrate, thereby offering a compelling evidence base for their advancement as phytotherapeutic candidates in reproductive toxicology and infertility management.

List of abbreviations

ANOVA: Analysis of Variance

Bax: Bcl-2-associated X protein (pro-apoptotic marker)

Bcl-2: B-cell lymphoma 2 (anti-apoptotic marker)

BW: Body Weight

C=O: Carbonyl group

C-H: Carbon-Hydrogen bond (aliphatic/aromatic)

C-O: Carbon-Oxygen bond (ester/ether)

C≡C: Alkyne group

C≡N: Nitrile group

CAT:Catalase

CC: Clomiphene Citrate

CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals

CYP: Cytochrome P450

Da: Dalton

DALY(s): Disability-Adjusted Life Year(s)

DNA: Deoxyribonucleic Acid

E2: Estrogen (Estradiol)

ELISA: Enzyme-Linked Immunosorbent Assay

ESI: Electrospray Ionization

FI: Fertility Index

FSH: Follicle-Stimulating Hormone

FTIR: Fourier Transform Infrared Spectroscopy

GBD: Global Burden of Disease

H&E:Haematoxylin and Eosin

HPG axis: Hypothalamic–Pituitary–Gonadal axis

HPLC: High-Performance Liquid Chromatography

IAEC: Institutional Animal Ethics Committee

IMI: Imidacloprid

K: Potassium

KBr: Potassium Bromide

LC-MS:Liquid Chromatography-Mass Spectrometry

LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry

LH: Luteinizing Hormone

MDA: Malondialdehyde

mg: Milligram

mL: Millilitre

m/z: Mass-to-Charge Ratio

MS: Mass Spectrometry

N_2: Nitrogen

Na: Sodium

ng/mL: Nanogram per Millilitre

NH_4^+: Ammonium Ion

NS:*Nigella sativa*

O-H: Hydroxyl group

OECD:Organisation for Economic Co-operation and Development

p.o.: Per os (oral administration)

P4: Progesterone

pg/mL: Picogram per Millilitre

ROS: Reactive Oxygen Species

rpm: Revolutions Per Minute

RT: Retention Time

SD: Standard Deviation

SEM: Standard Error of Mean

SOD: Superoxide Dismutase

TQ: Thymoquinone

UV: Ultraviolet

VAC / VC: *Vitex agnus-castus*

WHO: World Health Organization

µg: Microgram

µm: Micrometre

°C: Degree Celsius

Ethics approval and consent to participate:

This is a research article. Experimental rats are included in this study; the IAEC approval was obtained from CIP, Raipur, C.G.

Clinical trial: This is a research work, not a part of clinical trial. Hence, clinical trial number is not applicable.

Consent for publication:

This manuscript does not contain any personal data. Hence, no consent is required.

Conflict of interest:

The authors declare no conflicts of interest.

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Darshana Rathi: Preparation of content of the Manuscript

Poonam Sahu: Preparation of Diagram and figures.

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