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Research article

Untargeted metabolomics uncovers prime pathways linked to antibacterial action of citral against bacterial vaginosis-causing *Gardnerella vaginalis*: An *in vitro* and *in vivo* study

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ABSTRACT

Global increase in recurrence of bacterial vaginosis (BV) and worrisome rise in antimicrobial resistance pose an urgent call for new/novel antibacterial agents. In light of the circumstance, the present study demonstrates the *in vitro* and *in vivo* antibacterial activity of a phytochemical citral, with a particular emphasis to elucidate its mechanistic action against *Gardnerella vaginalis* –a potential cause of BV. Out of 21 phytochemicals screened initially against *G. vaginalis*, citral was envisaged to be a phenomenal antibacterial agent showing MIC and MBC at 128 μg/mL. Citral's rapid killing ability was revealed by a time-killing kinetics assay supported by CFU, signifying that it completely killed the given inoculum of planktonic *G. vaginalis* cells within 60 min. Further, citral was found to exhibit 1 min contact-killing efficacy together with mature-biofilm disintegrating ability at increasing MICs. To further understand the molecular action of citral, *in vitro* investigations such as ROS estimation, PI staining and intracellular protein release assay were performed, which demonstrated that citral deteriorated the membrane integrity of *G. vaginalis*. *Galleria mellonella*, a simple invertebrate model used to evaluate citral's non-toxic and antibacterial activity *in vivo*, demonstrates that citral completely restored the larvae from *G. vaginalis* infection. The metabolite level investigation using LC-MS revealed that citral had negative impact on biotin metabolism (*via.*, biotin), spermidine metabolism (*via.*, 5′-methyl-thioadenosine and spermidine) and nucleotide metabolism (*via.*, guanine, adenine and uridine). Since that biotin is associated with seven different metabolic pathways, it is conceivable that citral could target biotin biosynthesis or its metabolism and as a result, disrupt other metabolic pathways, such as lipid and fatty acid synthesis, which is essential for the creation of cell membranes. Thus, the current study is the first of its kind to delineate the promising *in vitro* and *in vivo* antibacterial efficacy of citral and decipher its plausible antibacterial action mechanism through metabolomic approach, which concomitantly emphasizes citral as a viable natural therapeutic alternative to manage and control BV.

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1. Introduction

Bacterial vaginosis (BV) is the most common non-inflammatory polymicrobial infection affecting women, particularly those of reproductive age, accounting for over 60% of all vaginal infections [1]. The clinical symptoms of BV include foul-smelling vaginal discharge, an increase in anaerobic bacteria, an increased pH in the vagina (due to an increase in amine production), and the presence of clue cells [2]. Besides, BV has also been associated with an increased risk of other clinical complications *viz*., intraamniotic infections, postoperative problems from infertility surgery and STIs [3]. Despite its high prevalence, the aetiology of BV remains poorly understood due to the wide diversity and complexity of microorganisms involved. Microbiologically, BV is a dysbiosis condition of vaginal microbiome, in which "good" lactobacilli are outcompeted by an abundance of anaerobic bacteria, including *Gardnerella vaginalis*, *Prevotella* spp., *Atopobium vaginae*, *Bacteroides* spp., and *Mobiluncus* spp [4].

In particular, *G. vaginalis* is postulated as most significant pathogen in the anaerobic bacterial consortiums of BV because it first adheres to the vaginal epithelium and then helps other anaerobes to attach and colonize for promoting polymicrobial biofilm interaction [5]. Unlike strict anaerobes, *G. vaginalis* is a facultative anaerobe and able to withstand the strong oxidation-reduction (redox) potential of a healthy vaginal microbiome. *G. vaginalis* is likely to promote the proliferation of additional stringent anaerobes by fostering a favorable microbiome environment with decreased redox potential, similar to the pathophysiology of facultative anaerobes in oral diseases [6].

A clinical study by Swidsinski et al. (2005) on vaginal biopsy specimens demonstrated that BV always exists with polymicrobial biofilm, with *G. vaginalis* being the primary component of the biofilm mass [7]. Besides, the vaginal human microbiome study found that *G. vaginalis* continues to be the major bacterial species in the vaginal microbiota of BV despite variations in the colonization by other BV associated anaerobic bacteria [8]. Recent research substantiate this fact that *G. vaginalis*, unlike other BV-associated anaerobes, plays a crucial role in BV pathogenesis by displaying high virulence traits such early adhesion, vaginal epithelial cell injury and apoptosis, and robust biofilm forming abilities [9,10]. Most crucially, *G. vaginalis* secretes a cholesterol-dependent cytolysin called vaginolysin, which helps with early adhesion by creating a cytotoxin specific to CD59 human complement regulatory molecule.

The common antibiotics typically used for the treatment of BV are clindamycin and metronidazole, and studies have shown that they are highly effective in roughly 75–86% of cases [11]. Despite the fact that these drugs may be initially helpful in removing pathogenic bacteria, they utterly fail to eradicate the biofilm architecture present in BV [6]. In particular, *G. vaginalis* biofilms are more resistant to these antibiotics treatments owing to the presence of extracellular polymeric substances and significant interspecies interactions within the biofilm matrix [12]. For instance, the study carried out by Qin et al. (2023) have demonstrated that the two strong biofilm forming strains (i.e., S20 & S23) exhibited resistance to the first-line BV drug, metronidazole, than the weak biofilm two strains (i.e., S24 & S25). They also found from RNA sequencing that Cas genes are highly up regulated during metronidazole treatment in biofilm producing strains, which further protect the cells from bactericidal effect of DNA-damaging agents, such as metronidazole [13]. Besides, according to Swidsinski et al. (2008), metronidazole therapy on *G. vaginalis* biofilms imposed mere temporary inhibition, and soon after the medication was stopped, the biofilm developments promptly restart [14]. As a result, *G. vaginalis* remains a major pathogen to be concerned in the both primary and recurrent of BV. Therefore, in order to overcome the drawbacks in the present antibiotic therapy, there exists a pressing need for the development of new/novel drugs against *G. vaginalis* with special emphasis on its biofilm production, which is crucial for the treatment and prevention of recurrent BV.

Due to its broad-spectrum biological activity and low toxicity, the use of plants as medicine is one of the oldest forms of medical practices and is now acknowledged as a viable strategy for battling diseases caused by bacteria that have developed a resistance to common antibiotics [15]. Traditionally, a variety of drugs and extracts derived from plants has been used to treat vaginal infections, either in combination or on their own [16]. For instance, Avicenna, a renowned Iranian scientist who lived from 980 to 1037 AD, claimed that lemongrass had therapeutic characteristics and can be used to treat vaginitis [16].

Citral is a key ingredient in lemongrass (*Cymbopogon citratus*), as well as in citrus fruits including oranges, limes, and lemon balm [17]. Over the past few decades, the extremely diverse pharmacological properties of citral including anti-hyperalgesic, anti-nociceptive, anti-inflammatory, antipyretic, anti-dyslipidemic, anti-diabetic and anti-adipogenic effects have been well-demonstrated by mounting body of researchers [18–21]. Notably, citral has also been reported to exhibit antimicrobial efficacy against other vaginal pathogens *viz*., C. *albicans*, *C. tropicalis* and *C. glabrata*. With this backdrop, the potent phytochemical -citral was selected in the present study to investigate antibacterial activity and *in vivo* protecting efficacy using a simple vertebrate model system *Galleria mellonella* against *G. vaginalis*.

Beyond identifying antimicrobials, it is critical to comprehend how they work, as this knowledge could be deployed to modify substances in a targeted manner in order to reduce host toxicity, increase affinity, or promote uptake, as well as create new targets for drug resistance [22]. Metabolomics is the study of metabolites, which provides a system-wide snapshot of the metabolites network in response to drug treatment; as a result, this method is frequently used to comprehend the molecular mechanistic action of antimicrobial treatments [23]. The current investigation uses an untargeted metabolomic approach based on LC-MS to determine the possible antibacterial mode of action on the planktonic proliferation of *G. vaginalis*.

2. Materials and methods

2.1. Strains and culture conditions

The test organism *G. vaginalis* (ATCC 14018) was purchased from HiMedia India. Tryptic soy broth (TSB) medium was used to culture and maintain *G. vaginalis*. TSB containing 1 % glucose and 0.5 % yeast extract (TSBgy) was used as a biofilm induction medium.

The log phase culture with 0.05 optical density (OD) (1×10^7 CFU/mL) was used as inoculum for all the *in vitro* assays.

2.2. Compound preparation

Totally, 21 phytochemicals *viz.*, citral, carvacrol, catechol, cinnamic acid, eugenol, citric acid, coumarin, pyrogallol, borneol, oleic acid, quercetin, curcumin, chlorogenic acid, ascorbic acid, gallic acid, linoleic acid, erucic acid, thymol, ricinoleic acid, phloroglucinol and cinnamaldehyde were used in the present study obtained from Sigma Aldrich (India). Metronidazole was used as positive control was purchased from Sigma Aldrich (India). The stock solutions were prepared at a final concentration of 100 mg/mL using ethanol as a solvent. Finally, the stocks were stored at 4 °C for further use.

2.3. Screening of phytochemicals against G. vaginalis planktonic growth

Aforementioned twenty-one phytochemicals were used in the screening of potential antibacterial agents against *G. vaginalis* [24,25]. Briefly, 1 % of *G. vaginalis* cultures (at OD600 = 0.05) was inoculated into 24 well microtiter plates (MTPs) containing 1 mL of TSB medium supplemented with 1024 μg/mL of respective phytochemicals. After an incubation period at 37 °C for 24 h in a 5% CO_2, the plates were read at 600 nm to determine the difference in the cell density.

2.4. Determination of minimal inhibitory concentration (MIC)

To determine MIC of citral against *G. vaginalis*, broth microdilution assay were performed [25]. In brief, *G. vaginalis* (1×10^7 cells/mL) was dispensed into 24 MTPs containing 1 mL of TSB medium supplemented with citral from 0 to 1024 μg/mL. Following 24 h incubation at 37 °C, OD at 600 nm was measured using a spectrophotometer (Spectra Max 3, Molecular Devices, United States). Here, metronidazole at varied concentrations (0–1024 μg/mL) was utilized to set as used as positive control. The lowest concentration of citral that exhibited visible growth inhibition in assay plate relative to the control was recorded as the MIC. All experiments were carried out in triplicates.

2.5. Determination of minimum bactericidal concentration (MBC)

In order to determine the MBC of citral, the spread plate method was followed as stated by Rodríguez-Melcón et al. (2021) with minimal modifications [26]. Following 24 h exposure with different concentrations of citral (0–1024 μg/mL), 100 μL of aliquots from the growth control (drug-free medium) and wells that displayed visible growth inhibition (an optically clear well) were plated onto TSB plates. Subsequent to incubation at 37 °C for 24 h, the minimal concentrations of citral that produced no colonies on agar plate was selected as MBC.

2.6. Time-kill kinetics assay of citral against G. vaginalis

The killing rate of citral against *G. vaginalis* planktonic growth were evaluated using previously described protocol by Swetha et al. (2020) with slight modifications [27]. In brief, 1 % of *G. vaginalis* (1×10^7 cells/mL) was added into the 5 mL tubes containing TSB medium supplemented with citral at MIC and 2XMIC. At predetermined time intervals (i.e., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h), 100 μL aliquots from each tube were used to measure the growth OD using a spectrophotometer. At the same time, 2 μL aliquots were used to spot on TSB plates for 10 h with 1 h time intervals. To evaluate the precise time of killing of citral, spot assay was carried out for 1 h with 10 min time intervals.

2.7. Citral's 1 min contact killing assay against G. vaginalis

The contact killing ability of citral at various concentrations was investigated by employing spot assay method described by Vincent et al. (2018) with little modifications [28]. Briefly, *G. vaginalis* (1×10^7 CFU/mL) was manifested with MIC, 2XMIC, 3XMIC, 4XMIC and 5XMIC of citral. A 2 μL aliquot was spotted on agar plates for 1 min with 15 s time intervals. The plates were stored at 37 °C for 24 h and imaged using a gel documentation system (GelDoc XR+, Bio-Rad, United States).

2.8. Post antimicrobial assay

G. vaginalis (1×10^7 CFU/mL) was subjected to short exposure with MIC and 2XMIC of citral for 30 min. Afterwards, the cell suspension was rinsed with PBS, centrifuged, and the supernatant was discarded to remove the citral. Then, 10 % culture from each group was inoculated into the fresh TSB medium. At predetermined time intervals (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h), the changes in the growth OD was measured at 600 nm [29].

2.9. Estimation of ROS production

To determine whether treatment with citral induce the production and accumulations of ROS in *G. vaginalis* cells, fluorescence microscopic analysis employing 2′,7′- dichlorofluorescein diacetate (DCFH-DA) was performed [30]. The *G. vaginalis* cells pretreated

with citral at MIC and 2XMIC were incubated for 20 min and pellets harvested by centrifugation, and then resuspended in 0.1 mL of PBS. After that, DCFH-DA was added at the final concentration of 10 μM and incubated at 30 °C for 30 min at dark. The changes in the fluorescence intensities of bacterial cells with and without citral exposure were examined under fluorescence microscopy. Subsequently, the suspension was read at the excitation of 485 nm and the emission of 535 nm.

2.10. Intracellular protein leakage experiment

To determine whether citral disrupts the cell membrane, leakage of intracellular protein to the extracellular medium was determined by performing cell leakage experiment [18]. *G.vaginalis* culture was exposed with citral at MIC and 2XMIC concentrations for 20 min. At predetermined time intervals (i.e., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h), 200 μL of aliquots was taken and the supernatant was incubated with Bradford reagent at dark, and read at 595 nm. Bovine Serum Albumin (BSA) at different concentrations was used as standard.

2.11. Effect of citral on premature and mature biofilm production of G. vaginalis

The potential influence of citral against *G. vaginalis* premature and mature biofilm formation was determined by crystal violet staining method according to the protocol described by Gowrishankar et al. (2019) with minor modifications [31]. For premature biofilm assay, overnight culture of *G. vaginalis* cells was added to 24 well MTPs containing 1 mL of TSBgy medium along with various concentrations of citral (0–1024 μg/mL) and incubated for 48 h at 37 °C without shaking to allow biofilm formation. For mature biofilm assay, the *G. vaginalis* biofilm was induced as stated above and treated with citral (MIC, 2XMIC, 5XMIC and 10XMIC) for 24 h. After removing the non-adherent planktonic cells, the adherent biofilm cells on the bottom were stained with 0.4 % crystal violet for 15 min, and then destained with 15 % glacial acetic acid. The formed biofilms were quantified by reading at 570 nm. The percentage of biofilm inhibition was calculated using the following formula.

The relative biofilm inhibition: 100 × (biofilm formation of untreated control – biofilm formation of treated/biofilm formation of untreated control).

2.12. In vivo toxicity and efficacy evaluation on Galleria mellonella larvae

The *in vivo* toxicity and efficacy of citral was assessed using the invertebrate animal model *G. mellonella* [32]. Larvae weighing approximately 0.2–0.4 g were chosen for this experiment. Larvae were divided randomly into the following six different groups (five larva/group), *viz*., Group I: Naive control; Group II: Toxicity control-1 (citral at MIC); Group III: Toxicity control-2 (citral at 2XMIC); Group IV: Infection control (*G. vaginalis*); Group V: Treatment group-1 (*G. vaginalis* + citral at MIC) and; Group VI: Treatment group-2 (*G. vaginalis* + citral at 2CMIC). Bacterial culture and drug were injected using a U-100 insulin syringe in the left and right last prolegs, respectively. Uninjected larvae serve as the naive group. Post inoculation, larva groups were incubated at 37 °C for 5 days and monitored for their survival for every 24 h.

2.13. Histopathological analysis

To evaluate citral's efficiency against *G. vaginalis* infections, histopathological studies were performed. The larvae were processed for histology three days after post inoculation/treatment. The sliced tissue sections of larva from all groups were further stained with hematoxylin and morphological changes, if any, were visualized under light microscopy [33].

2.14. Preparation of metabolites for LC-MS analysis

The total cellular metabolites of *G. vaginalis* were extracted in the presence and absence of citral by following the protocol described by Meza-Villezcas et al. (2022) with little modifications [34]. In brief, 15 mL of log phase culture of *G. vaginalis* cells (OD 0.8) treated with citral at 64 μg/mL (sub-MIC of citral) for 1 h at 37 °C. The TSB medium containing *G. vaginalis* culture devoid of citral was considered as control. Following incubation, the cell pellet was collected by centrifugation at 3220 *g* at 4 °C and cell density was adjusted to OD 1 by resuspending with PBS. The cell pellets were rinsed three times with PBS (4 °C) and centrifuged for 3 min at 3220×*g* at 4 °C. The pellet was air dried and stored at −80 °C for further processing. The metabolites extracted from the dried sample with methanol:acetonitrile:ethyl acetate at (1:1:1 ratio) by sonication. Following that, samples were centrifuged at 4 °C for 10 min at 14,000 g. The supernatant was transferred to a 1.5 mL Eppendorf tube, and allowed to air dry at room temperature. The dried extracts were resuspended in an 80:20 v/v water and acetonitrile solution and centrifuged at 14,000 g for 10 min at 4 °C, to collect particle-free supernatant for LC-MS analysis. Quality control (QC) samples are made by combining equal amounts of all extracted samples.

2.15. LC- QTOF-MS analysis

LC–MS analyses were performed on a LC-QTOF (Agilent). Water and acetonitrile with 0.1 % formic acid were considered as mobile phase at a flow rate of 300 nL/min. The gradient system increases from 5 % B to 20 % B linearly for 15 min, then maintains at 20 % B for 10 min before increasing to 100 % B in 1 min and maintaining at 8 min before the next sample [35]. The injection sample volume was 10 μL with a run time of 32 min. To reduce any carry-over, two blank samples (3 μL of mobile phases A and B at a 95:5 ratio) were run

between the experimental sample injections. The MS system was operated under positive electro-spray ionization (ESI). The chromatographic peaks, signal reproducibility and analyze stability were all checked by analyzing pooled QC samples at regular intervals throughout the batch.

2.16. Data processing and analysis

The metabolite data was processed using MetaboAnalyst 5.0. In brief, missing values in the raw data were first checked, and any metabolite with more than 50 % missing values was excluded from further analysis. To create a Gaussian data distribution for additional statistical analysis, the raw data was log-transformed and automatically scaled. Unsupervised PCA (principal component analysis) and supervised PLS-DA were used to examine differences in the metabolomics profiles of samples (partial least-squares discriminant analysis). One-way analysis of variance (ANOVA)-based cross-validated P-value significance was set to P 0.05. The significant metabolite changes (≥1.5- fold changes) between the groups identified using multiple comparisons, *post hoc* analysis employed by Tukey's Honestly Significant Difference (Tukey's HSD). Pathway enrichment analysis was processed in the enrichment analysis module [36].

2.17. Statistical analysis

All the experiments were carried out in biological triplicates with at least two experimental replicates and the data were presented as mean ± standard deviation. To evaluate statistical differences between control and treated samples one-way analysis of variance (ANOVA) and Dunnett's post hoc test was performed using SPSS statistical software 17.0. The significance was represented as $p \leq 0.05$ and < 0.01, respectively.

3. Results and discussion

With the consequence of antibiotic-centered treatment, there is a significant rate of recurrence of BV—approximately 60% within six months after treatment [37]. Most notably, biofilm states of *G. vaginalis* display extremely high resistance to conventional antibiotics, indicating that this feature is an important pathogenic trait in the pathogenesis of BV. As a result, developing novel therapeutic techniques, particularly those effective against *G. vaginalis* and its associated biofilm, has become an urgent necessity for treating BV.

Essential oils (EOs) are complex combinations of non-toxic; low-molecular-weight volatile chemicals extracted from aromatic plants and have been used medicinally for thousands of years [38]. They have been recognized for their great therapeutic properties including antibacterial, antifungal, antioxidant and antiviral effects [39–41]. Some of the investigation tested the promising antimicrobial activity of EOs on VVC [42], BV [43], trichomoniasis vaginitis (TV) [44]. However, as far as we know, relatively very limited studies have shown that EOs can be used to treat vaginal infections. One of the main disadvantages of using EOs is that fluctuations of their composition depending on collection, which may result in changes in their activity [45]. As a result, studying more about the

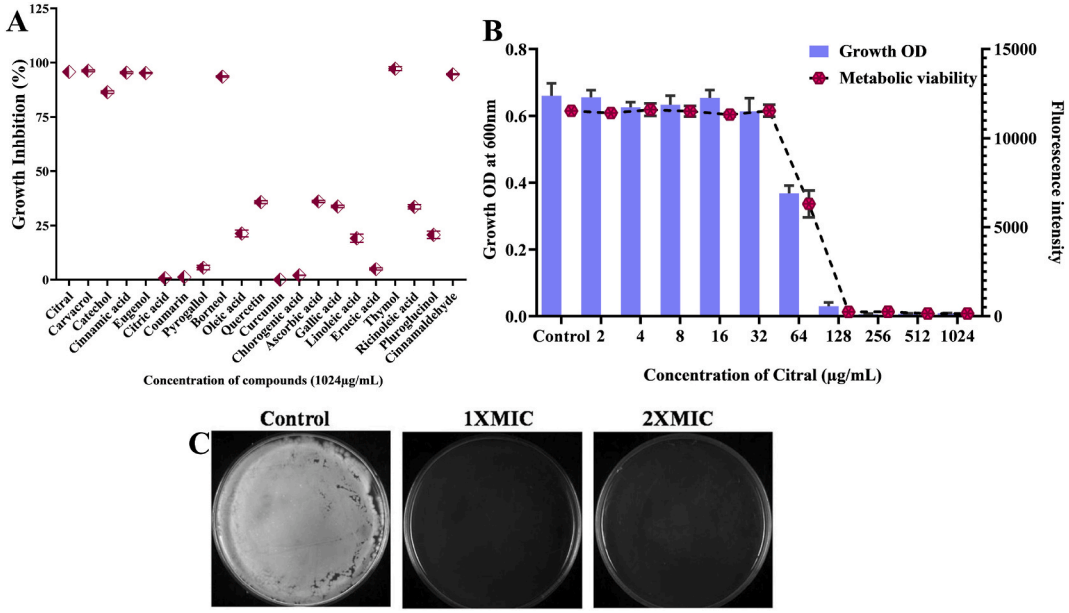

Fig. 1. Screening of phytochemicals and evaluation of citral MIC. (A) Screening of 21 phytochemicals for their inhibitory potential on *G. vaginalis* planktonic growth at 1024 µg/mL. **(B)** Determination of citral MIC and its influence on the true metabolic viability of *G. vaginalis* using broth microdilution and alamar blue assay, respectively. **(C)** Determination of MBC of citral through spread plate method.

individual chemical components found in EOs can offer new insights to understand their antimicrobial activity.

Cymbopogon Citratus is one of the most important medicinal plants, and is used mostly in aromatherapy as essential oil, particularly, in Southeast Asia. This plant was claimed for its renowned therapeutic properties to treat vaginitis by Avicenna, a great Iranian scientist who lived from 980 to 1037 AD [16]. Citral, which is abundant in *Cymbopogon Citratus*, has long been known for its medicinal characteristics, including anti-hyperalgesic, anti-nociceptive, anti-inflammatory, antipyretic, anti-dyslipidemic, anti-diabetic, and anti-adipogenic activities [[18–21]]. Against this backdrop, current study investigates the antibacterial activity and *in vivo* protecting efficacy of citral using a simple vertebrate model system *Galleria mellonella* against an important vaginal pathogen- *G. vaginalis*. Also, this work uses LC-MS-based metabolomics techniques to decipher the mechanistic impact of citral on the planktonic proliferation of *G. vaginalis*.

Initial screening of 21 phytochemicals revealed seven potential antibacterial agents *viz*., citral, cinnamaldehyde, borneol, eugenol, carvacrol, thymol and catechol against *G. vaginalis* planktonic growth (Fig. 1A). To further scrutinize the best antibacterial compound from these 7, their MIC was evaluated. The identified MICs of seven phytochemicals (citral, carvacrol, cinnamic acid, eugenol, borneol, thymol and cinnamaldehyde) and positive control metronidazole are illustrated in Table 1. Citral has the lowest MIC value among these seven potential hits (Table 1). Also, there are currently no reports on the antibacterial activity of citral against *G. vaginalis*. Based on this, citral has been selected for further investigation.

The obtained growth OD and metabolic viability of *G. vaginalis* in the treatment with various concentrations i.e., 0–1024 μg/mL of citral is shown in Fig. 1B. The MIC and MBC were both determined to be 128 μg/mL, indicating that citral's antibacterial properties are neither bactericidal nor bacteriostatic (Fig. 1B and C). The positive control metronidazole MIC was found to be 128 μg/mL as same as citral against *G. vaginalis* 14018. Alamar blue assay was used to determine *G. vaginalis*'s actual metabolic viability following citral treatment. According to the resazurin-assisted assay, citral had the greatest impact on the metabolic viability of *G. vaginalis* between 128 and 1024 μg/mL, which was in consistent with the results of the broth microdilution experiment (MIC assay) (Fig. 1B).

Based on the MIC result, the killing rate of citral at various time intervals was evaluated by subjecting *G. vaginalis* cells to spectrometric and spot assay assisted time killing kinetics study. Initially, the time-killing kinetics study was conducted for 10 h with 1 h time interval, which revealed that citral at MIC effectively killed all *G. vaginalis* cells within 1 h exposure (Fig. 2A and B). In order to determine the exact time of killing, the kinetic assay was further conducted for 1 h with 10 min time intervals, which demonstrates that citral at MIC and 2XMIC completely eradicated the *G. vaginalis* cells at 60 and 10 min, respectively (Fig. 2C). It was evident from the time-killing kinetics that the concentration of the drug compound is the only factor that determines the bactericidal activity of any compound. The findings of this experiment are in congruence with those of Leite et al.'s (2014) investigation [46], which revealed that citral's fungicidal properties increased as citral concentration increased.

The "contact killing" or "rapid microcidal" capabilities of antimicrobial drugs accelerate the eradication of harmful bacteria from the environment [28]. As citral was intended to be employed in various clinical formulations for disease prevention, the contact killing activity of citral at different concentrations (1X, 2X, 3X, 4X & 5XMIC) was evaluated using a 1-min contact killing assay. The result demonstrated that citral from 3XMIC had enough potential to completely eradicate *G. vaginalis* cells within a min exposure (Fig. 3).

The term post antibiotic effect (PAE) refers to the length of the time taken for the target microorganism to regenerate following transient antibiotic treatment, which is essential for the antibiotics to determine its dosage frequency in clinical settings [29]. The data from PAE indicated that a brief exposure to citral at MIC was effective in preventing the growth of *G. vaginalis* for up to 5 h. On the other hand, citral at 2X, 3X, 5X and 10XMIC had completely inhibited *G. vaginalis* growth, and hence, there was no regrowth observed even after 10 h incubation. This finding is well in accordance with the outcomes of spot and spectrometric assisted time killing kinetics assays.

3.1. Citral (at MIC) induces the cell membrane damage

Cell wall integrity is crucial for the survival of bacterial cell under stressed conditions. Disruption of cell membrane integrity causes the efflux of cytoplasmic constituents to the outer environment [47]. In order to know whether citral exposure impose any changes in the cell membrane integrity, intracellular proteins released to the extracellular environment were quantified. As anticipated, the increased intracellular protein absorbance following treatment of G. vaginalis with different concentrations of citral (MIC and 2XMIC) clearly demonstrates increased cell membrane permeability following citral exposure (Fig. 4D). During 2XMIC and MIC treatments, there was a sudden increase in protein release at 1 and 2 h, respectively. After that, no appreciable increase in the protein release was

Table 1
MIC of identified phytochemicals against *G. vaginalis* growth.

Phytocompounds	MIC against *G. vaginalis*
Citral	128
Carvacrol	256
Borneol	512
Cinnamaldehyde	512
Thymol	256
Eugenol	512
Cinnamic acid	512
Metronidazole	128

The MIC result are expressed as mode in μg/mL.

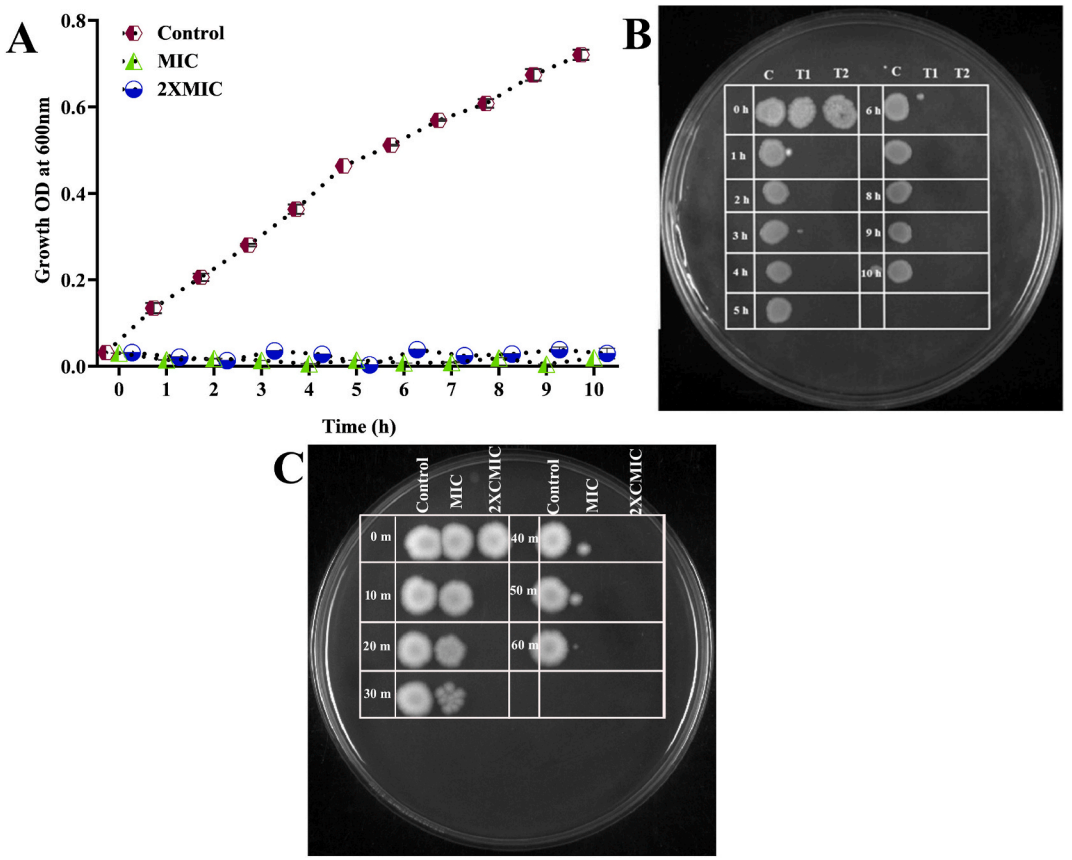

Fig. 2. Time killing kinetics of citral on *G. vaginalis* planktonic growth. **(A)** Evaluation of killing rate of citral via CFU assisted time killing kinetics. **(B)** Spot assay revealed the killing nature of citral against *G. vaginalis* for 10 h with 1 h time intervals. **(C)** Spot assay carried out for 1 h with 10 min time intervals to find the exact killing time of citral.

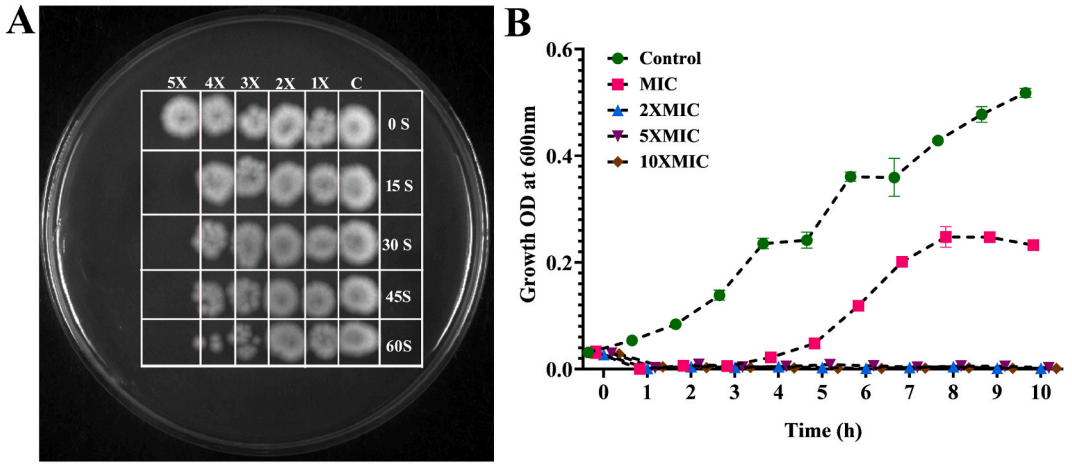

Fig. 3. **(A)** Spot assay revealing the contact killing efficiency of citral at various concentrations (MIC, 2XMIC, 3XMIC, 4XMIC & 5XMIC) with in 1 min span of exposure with *G. vaginalis*. **(B)** PAE of citral evaluated by spectrometric method.

observed. This supports the speculation that citral damages cell membranes of all *G. vaginalis* cells within a very short exposure period. The observations of this experiment are in good agreement with those of Wang et al. (2019) [48] and Shi et al. (2016) [18], who showed that citral manifestation damages cell membranes as evidenced by increased intracellular protein leakage.

Further, fluorescence microscopic investigation with PI dye assistance was carried out to better understand the citral-mediated loss

of cell membrane integrity. PI is considered an indicator of cell membrane integrity, as it can specifically penetrates and stains DNA in dead cells or those with compromised cell membranes [49]. As a result, cells with damaged membranes easily allow PI to enter and bind DNA, resulting in an increase in fluorescence intensity. The fluorescence micrograph of citral-treated cells revealed an increase in fluorescence intensity when compared to the untreated control (Fig. 4C). In addition, it was apparent that the fluorescence intensity increased with an increase in citral concentration in treated wells. The results of this experiment, together with a protein leakage experiment, substantiated citral-mediated cell membrane damage in *G. vaginalis* cells. The present experiment's findings were quite similar to those of Wang et al. (2018) [49], which demonstrated that *Penicillium expansum* membrane integrity was lost because of increasing citral concentrations, as demonstrated by PI staining. A mounting body of other investigations have also shown that the pathogens *Cronobacter sakazakii* [18], *Alternaria alternative* [48], *Enterobacter cloacae* [50], and suffer citral-mediated cell membrane damage. It is inferred from these studies that, citral could result in a loss of cell membrane integrity that causes cell death in order to combat different diseases.

3.2. Citral treatment does not impose oxidative stress in G. vaginalis cells

ROS production and accumulation within cells disrupt the cellular redox equilibrium, which may further result in deterioration of cellular components [51]. A plethora of reports have documented that EO interferes with the homeostasis of oxidative stress and imbalance of ROS to promote cell death in various pathogens [52,53]. Therefore, the accumulation of ROS upon citral treatment was evaluated. There was no observable difference in fluorescence intensity between the treated and untreated control cells (Fig. 4A and B), indicating that citral treatment does not cause ROS accumulation in *G. vaginalis* to endorse cell death.

3.3. Citral potentially eradicates the mature biofilm production of G. vaginalis

The ability of microbes to form biofilms on a variety of surfaces has been an efficient strategy to evade host immune response and antimicrobials' action. Earlier research on BV demonstrated that there is a complex interaction between pathogenic species in the vaginal niche, predominantly mediated by *G. vaginalis* biofilm formation, which results in a polymicrobial biofilm formation [7]. With its innate capacity for strong adhesion and biofilm formation properties, *G. vaginalis* plays a crucial role in the pathogenesis of BV by adhering to the vaginal epithelium and facilitating the attachment of other BV related species [7,54]. Therefore, the possible influence of citral on the architecture of *G. vaginalis* biofilm was evaluated by employing the crystal violet staining and light microscopic methods. The potency of citral to suppress *G. vaginalis*'s premature biofilm development was initially tested, and the results revealed that citral was ineffective at sub-MIC levels (Fig. 5A). Citral exhibits biofilm-inhibitory potency at its MIC and above by impairing cellular viability (Fig. 5A), indicating that citral does not obey the ideal concept of an antibiofilm agent, i.e., biofilm inhibition at non-lethal concentrations.

On the other hand, spectrometric measurement of the pre-formed biofilm of *G. vaginalis* revealed that citral at MIC and 2XMIC

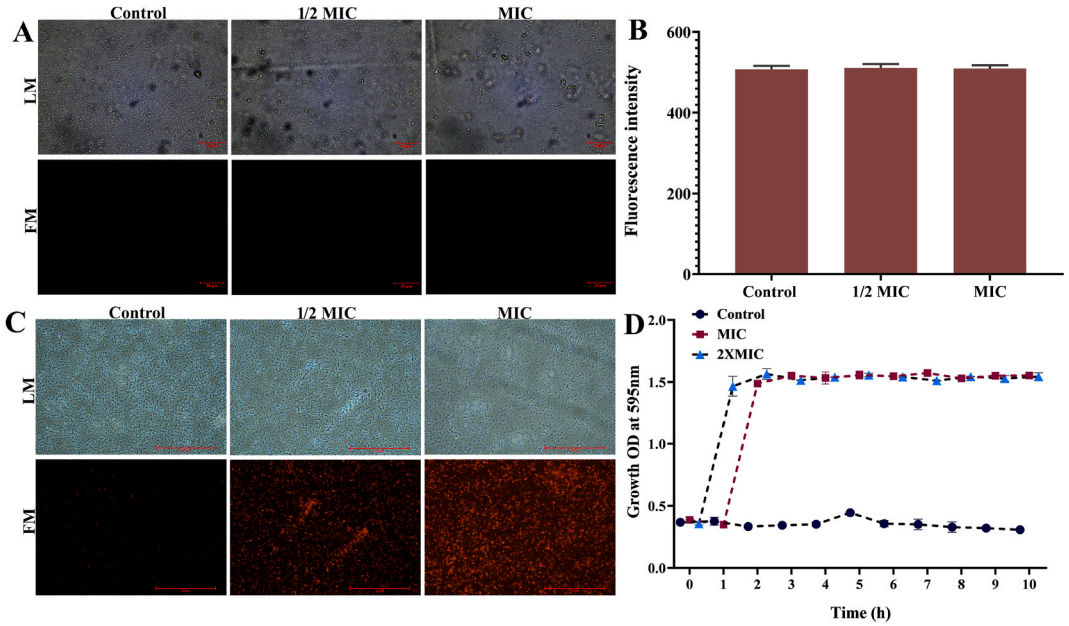

Fig. 4. Elucidating the mode of killing of citral. (**A & B**) Assessment of ROS accumulation during citral treatment (1/2 MIC & MIC) via **(A)** fluorescence microscopic and **(B)** spectrometric method **(C)** Assessment of cell membrane integrity loss in *G. vaginalis* upon citral manifestation using PI staining. **(D)** Intracellular protein release quantified at pre determined time intervals after exposing *G. vaginalis* to citral treatment (at MIC and 2XMIC).

effectively eradicated the preformed-biofilm with a percentage ranging from 75 to 95 (Fig. 5B). Furthermore, there was no discernible difference in the effectiveness of eradicating pre-formed biofilm when the citral concentration was increased to 5X and 10XMIC compared to 2XMIC. Aside from that, just 25 % of the pre-formed biofilm was observed to be eradicated by the citral sub-MIC (Fig. 5B).

Citral-induced biofilm disruption was further ascertained through light microscopic examination, wherein, the disintegration of pre-formed biofilm was quite apparent in the citral treated micrographs compared to the untreated control micrographs (Fig. 5C). While, the micrograph of untreated control displayed the hallmark biofilm architectural characteristic features *viz.*, highly aggregated network of complex, multilayered biofilm cells (Fig. 5C).

3.4. *In vivo assays with G. mellonella revealed the non-toxic nature and promising efficacy of citral*

Despite of citral's efficacious nature against *G. vaginalis*, its non-toxic nature under *in vivo* conditions should be guaranteed prior to its usage as therapeutic formulations for biomedical applications. Hence, a *G. mellonella in vivo* model was used to evaluate the toxicity of citral. Off late, *G. mellonella* has been grabbing the attention of researchers working in the arena of therapeutics, particularly for *in vivo* toxicity assessment of newly identified bioactives/antimicrobials, owing to its high degree of homology with mammalian cells [55].

In vivo efficacies of antimicrobial drugs against vaginal pathogens, particularly *C. albicans* infections, have been studied using the *G. mellonella* model system [56,57]. However, there are no reports on using *G. mellonella* as a model to study/research *G. vaginalis* infection. Herein, we used *G. mellonella* larvae to determine citral's *in vivo* effectiveness against *G. vaginalis* infections. In this study, *G. mellonella* is not employed as a model for vaginal infections, but rather as *in vivo* model to assess effectiveness of citral against *G. vaginalis* under *in vivo* condition. The survival of larvae in the entire experimental groups was monitored for 5 days.

Data on *in vivo* toxicity demonstrated a 100% survival rate in both the experimental groups (5 larvae/group) i.e., (i) larvae exposed to citral at MIC and (ii) 2XMIC even after five days of inoculation, signifying the non-toxic nature of citral under *in vivo* condition (Fig. 6A and C).

Similarly, data on *in vivo* efficacy evaluation of citral against *G. vaginalis* infected *G. mellonella* larvae demonstrated that citral treatment at MIC and 2XMIC prolonged the survivability of *G. vaginalis* infected larvae to the range of 60 and 80 %, respectively (Fig. 6A and C). Whereas, the infection control group displayed 0% survival within 4 days post-inoculation (Fig. 6A and C).

Furthermore, the efficacy of citral in reducing the bacterial load under *in vivo* condition was evaluated by bacterial burden analysis. The bacterial load calculated at 0 and 3rd day of post-inoculation with *G. vaginalis* and citral. The results depicted that the bacterial load appeared to be two times higher in the infection control group after 3 days than at 0 day of inoculation, which illustrates the awful

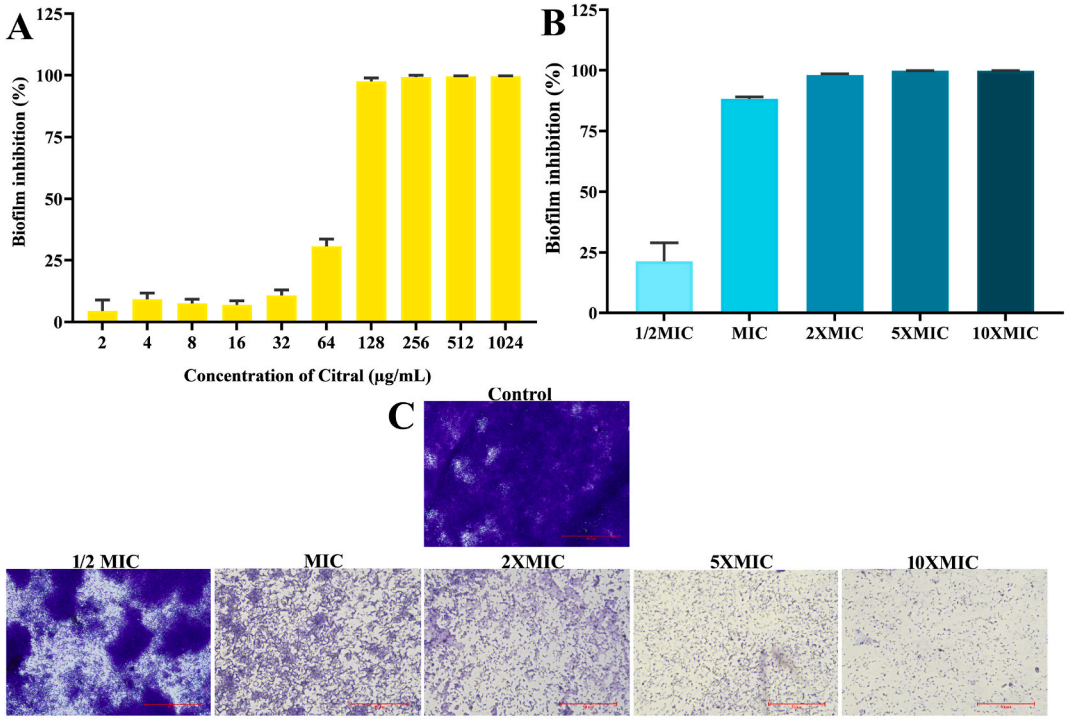

Fig. 5. Influence of citral on premature and mature biofilm production of *G. vaginalis*. **(A)** Impact of citral at various concentrations (0–1024 µg/mL) on *G. vaginalis* premature biofilm formation, quantified by crystal violet staining method. **(B)** Spectrometric quantification reveals the mature biofilm eradication ability of citral at MIC, 2X, 5X, 10XMIC. **(C)** Fluorescence micrographs displaying the disintegration of *G. vaginalis* mature biofilm production during citral manifestation.

pathophysiology of *G. vaginalis* and infection establishment in *G. mellonella*. On the other hand, citral (2XMIC) manifestation, drastically reduced the bacterial burden by twofold when compared to the untreated infection control (Fig. 6B). Despite this, citral at MIC was ineffective in lowering bacterial load, since the bacterial load appears to be comparable to that of the infection control group (III) (Fig. 6B).

Further, a histopathology investigation was carried out to unravel antibacterial efficiency of citral at the tissue level. After 3 days of post inoculation, one animal from each group was sacrificed for histopathological analysis. Microscopic examination of microtome-tissue smears revealed no abnormalities in the naive control and the toxicity control (citral at MIC & 2XMIC), confirming citral's non-toxic nature *in vivo* (Fig. 7). This is in line with the results of the survival assay (Fig. 6A), which showed no apparent differences between the survivability of larvae exposed to citral and naive controls.

Nonetheless, *G. vaginalis*-infected larvae groups showed a variety of tissue abnormalities, including hemocyte infiltration, micro vacuolization, multifocal melanization, and nodule development to the various tissue sites of larvae such as the fatbody, paratracheal regions and stomach (Fig. 7).

On the other hand, treatment with citral at 2XMIC completely recovered the larvae from *G. vaginalis*-induced tissue damage, as evidenced by the production of highly decreased nodules, multifocal melanization, and micro vacuolization (Fig. 7). The disintegration of hemocyte dispersion to maintain tissue homeostasis and complete recovery of larvae tissue after citral treatment was evident by the absence of hemocyte infiltration. Even though the treatment with citral at MIC showcases efficacy in preventing tissue damage induced by *G. vaginalis*, the complete recovery has not been observed, as apparent through the presence of few number of haemocytes and melanization (Fig. 7).

3.5. *Untargeted metabolomic analysis signifies the impact of citral on basic metabolic pathways of G. vaginalis*

Metabolomics has been utilized to gain a simplified overview on the cellular process under defined conditions, which provides a new platform to comprehensively understand the microbial physiology and mechanistic action of new drugs in the *de novo* discovery process [57,58]. Moreover, understanding drug resistant mechanisms of microbial pathogens in response to antimicrobial drug treatment through metabolomics approach can potentially offer the development of alternative treatments with new drug targets [23]. To comprehend the mechanistic action and potential drug targets of citral in *G. vaginalis*, untargeted metabolomic analysis was performed using LC-MS. Three independent biological replicates of control and citral treated samples were prepared and analyzed through a single LC-MS batch. After removing the redundancy of identified compounds (through Medline library search) from LC-MS, 1081 metabolites have been obtained in both control and citral treated samples (Fig. 9A). After removing the missing feature (<50% missing value), 570 metabolites were taken further for multivariate and univariate analysis (Fig. 9B).

A multivariate statistical analysis has been carried out to observe the metabolite changes in citral treated samples compared to that of untreated control samples. In Principal component analysis (PCA), the control and citral treated samples have minimal variations in

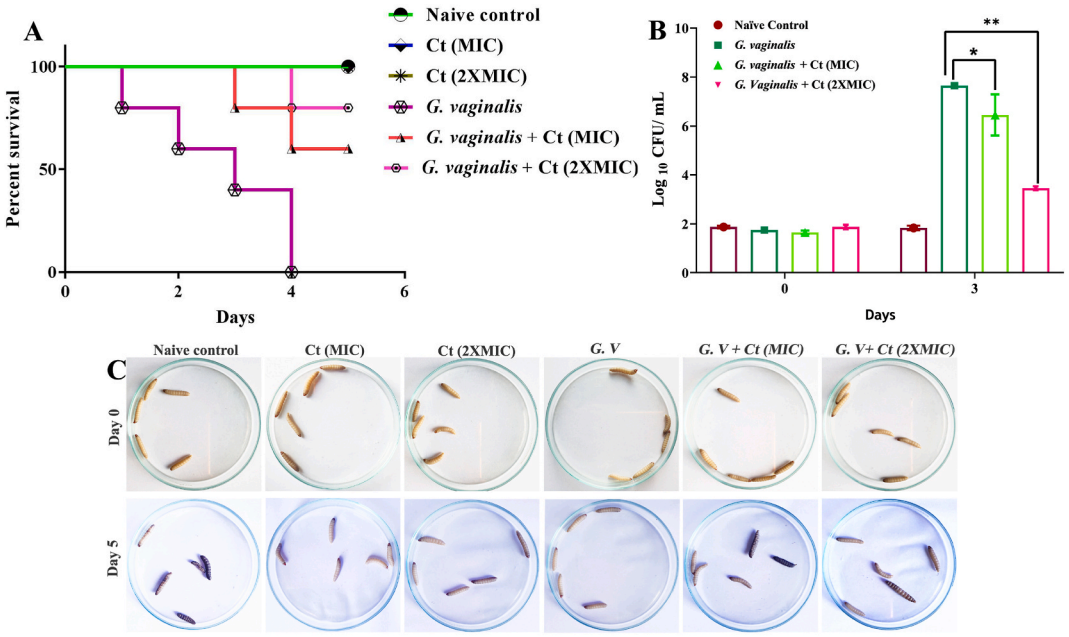

Fig. 6. *In vivo* toxicity and efficacy evaluation of citral. **(A)** Survival rate of larvae exposed with and without citral at MIC and 2XMIC in the presence *G. vaginalis* infections over 5 days of incubation period. **(B)** Bacterial burden analysis before (at day 0) and after (at day 3) treatment with citral at MIC and 2XMIC during *G. vaginalis* infections. **(C)** Representative image displaying the survivability of larvae exposed to *G. vaginalis* and citral treatment. Death of larave is indicated by black color formation and shrunken of body mass. White colored larvae represent good healthy.

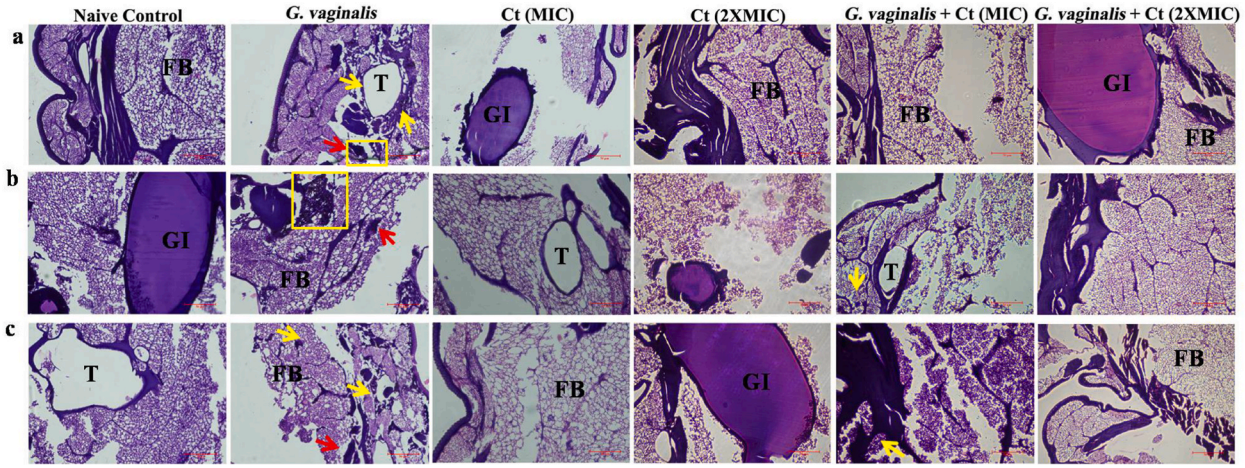

Fig. 7. Histopathological analysis showcasing the appearance of larvae tissues in the presence and absence of citral treatment during *G. vaginalis* infection. The tissue of larvae inoculate *G. vaginalis* represents the hemocyte recruitment (yellow colored arrow), melonization (red colored arrow) along with nodules formation (yellow colored square box). **a, b, c** represents the three different location of same larvae obtained from single experiments. **Ct**-cuticle; **FB**- fat body; **GI**- gastrointestinal tract; **T**-trachea.

their three biological replicates, revealing the minimal technical variations within the group (Fig. 8A). In addition, control and citral treated groups were completely separated from each other and form two distinct clusters, confirming the global metabolic differences between two groups.

In agreement with PCA, the supervised extension of PCA i.e., PLS-DA analysis indicated a substantial difference between the untreated control and citral-treated groups, demonstrating the pronounced changes in *G. vaginalis* metabolic profiles after citral treatment (Fig. 8B). In the variable importance for projection (VIP) analysis of PLS-DA models, metabolites with VIP values greater than one significantly aided in distinguishing the control from treatment groups (Fig. 8C).

Further, univariate analysis ($\geq$1.5-$\log_2$-fold; ANOVA, $p \leq 0.05$, FDR $\leq$0.1) was used to identify the differentially regulated metabolites between groups. Overall, 35.43 % (202 metabolites) of significantly altered metabolites ($\geq$1.5-$\log_2$-fold; ANOVA, $p \leq 0.05$, FDR $\leq$0.1) in citral treated samples was observed. Fig. 2, showcases the significantly downregulated, up-regulated and unchanged/unaltered metabolites in the citral treated samples (Figure C).

To further study the exact metabolic pathways by which citral impacts *G. vaginalis* planktonic cells, the biological function of identified differentially regulated metabolites (DRMs) was assessed using a KEGG pathway impact analysis. As shown in Fig. 10, the identified DRMs were associated to polyamine production (spermidine and spermine biosynthesis), amino acid metabolism (arginine, alanine, glutamate, and proline metabolism), amino acid degradation, fatty acid biosynthesis, nucleotide metabolism (purine and pyrimidine metabolism) and basic metabolic pathways (citric acid cycle, gluconeogenesis, warburg effect) in citral treated *G. vaginalis* cells. Out of 17 identified pathways, biotin metabolism as well as spermidine & spermine biosynthesis have high impact ratio of 16 and 14.18, respectively. Valine, leucine and isoleucine degradation pathway was found to have low impact ratio such as 2.132 (Fig. 10).

According to the enrichment study, one of the DRMs -biotin was linked to seven metabolic pathways, including biotin metabolism (16 impact), alanine metabolism (7.519 impact), citric acid cycle (4 impact), ammonia recycling (4 impact), gluconeogenesis (3.663 impact), fatty acid biosynthesis (3.663 impact), pyruvate metabolism (2.667 impact), valine, leucine, and isoleucine degradation (2.132 impact) (Fig. 10).

Biotin, often known as vitamin B7, is essentially required for several metabolic pathways in bacteria, including the tricarboxylic acid cycle and amino acid metabolism. In all three domains of life, this metabolite is regarded as a necessary amino acid. However, the *de novo* synthesis of biotin is only found in microbes, plants and some fungi; humans lack this metabolic pathway, therefore they must obtain biotin from dietary sources or intestinal biotin-producing bacteria [59]. This makes biotin production a top target for the development of novel drugs to combat pathogenic bacteria.

In the present work, biotin metabolism was found to be downregulated in cells treated with citral, suggesting that citral may target biotin metabolism and consequently influence other metabolic pathways. According to certain research, biotin plays an important role in lipid production, which is a key component of bacterial cell membranes [60,61]. Our *in vitro* investigation using an intracellular protein leakage assay and PI staining divulges the cell membrane damage and membrane integrity loss during citral treatment in *G. vaginalis*.

In light of the combined findings of untargeted metabolomic analysis and *in vitro* experiments, it is highly pertinent to comprehend that citral, by potentially targeting biotin synthesis and metabolism, influences other metabolic pathways, including the production of lipids and fatty acids, which are essential for the formation of cell membranes. The impacted biotin metabolism and the associated metabolic pathways are showcased in Fig. 11.

Next to biotin metabolism, polyamine metabolisms such as spermidine and spermine were highly impacted (14.18 impact) during citral treatment. Several studies documented that polyamines, especially spermidine, is highly essential in archaea and eukaryotes

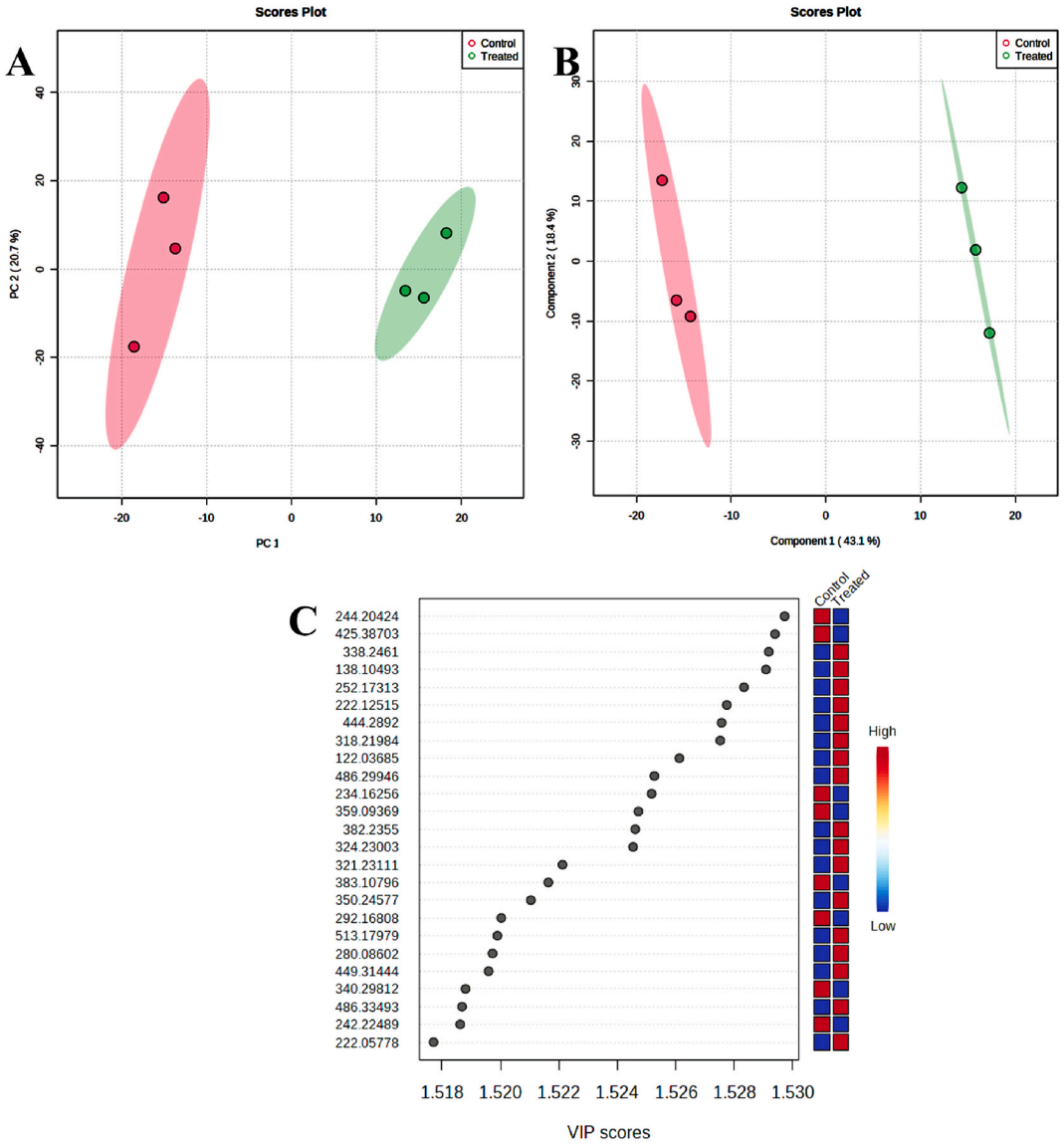

Fig. 8. Multivariate statistical analysis. **(A)** Principal Component Analysis (PCA) using the aligned and filtered features among citral treated and untreated groups. **(B)** Partial least squares discriminant analysis (PLS-DA) score plot component 1 (43.1% and component 2 (18.4%). Each dot stands for a separate biological copy. The treated group is denoted by green spots, while the control group is denoted by red dots.

[62]. The function of polyamines includes transcription, translation, cell growth stimulation and most importantly biofilm formation [63]. In particular, spermidine has been reported to be essential for planktonic growth of some Gram-negative bacteria, such as *Campylobacter jejuni* and *Pseudomonas aeruginosa* [64]. In the present study, citral treatment was found to significantly down-regulate two metabolites that are involved in spermidine and spermine metabolisms *viz.*, 5′-methylthioadenosine and spermidine. Notably, these two metabolites were also linked with methionine metabolism.

Furthermore, citral exposure may have downregulated the levels of nucleotides linked with purine and pyrimidine metabolism, such as guanine, adenine, and uridine. Altogether, it has been observed that citral manifestation induced down-regulation of several metabolites that were linked with numerous basic metabolic pathways, resulting in halted metabolic function essential for the survival of planktonic cells of *G. vaginalis*. The overall impact of identified DRMs on the metabolic pathway of *G. vaginalis* were mapped using *G. vaginalis* KEGG database (Fig. 12), which shows citral treatment impact the basic metabolic pathways such as nucleotide, carbohydrate, lipid, amino acid and energy in *G. vaginalis* cells.

4. Conclusion

Alternative antimicrobial therapies must be urgently researched due to the rise in chronic infections that are resistant to current

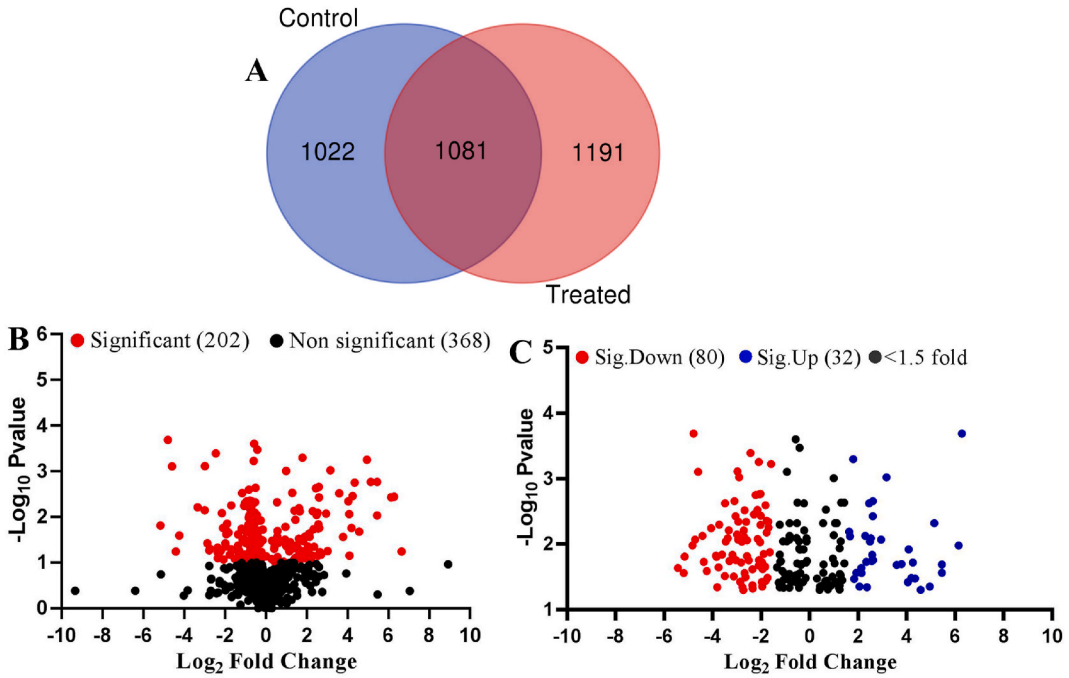

Fig. 9. Significant and non-significant of identified metabolites. **(A)** Ven diagram showcasing the obtained and common metabolites between citral treated and untreated samples. **(B)** Volcano plot demonstrating the statically significant and non-significant obtained metabolites after removing the missing feature (<50 % missing value). Red and black colored dots in the volcano plot denotes significant and non-significant metabolites. **(C)** Volcano plot showcasing the downregulated and upregulated significant metabolites. Red, blue and black colored points indicates downregulated, upregulated and unchanged significant metabolites.

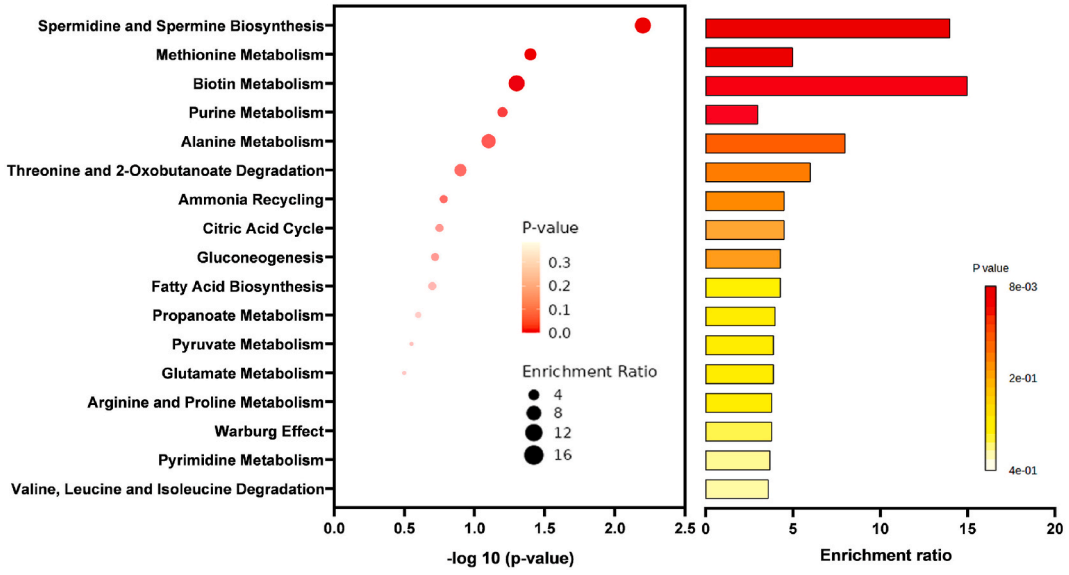

Fig. 10. The top 25 putative metabolic pathways impacted by citral treatment were assessed using enrichment analysis. Using differently regulated metabolites, enrichment analysis was performed using MetaboAnalyst 5.0 software with reference set extracted from SDPDB database.

antibiotics. The current investigation shows that citral has antibacterial action against *G. vaginalis* -a significant vaginal pathogen causing BV. Citral was found to exhibit phenomenal antibacterial as well as pre-formed biofilm disrupting efficacies against planktonic and mature biofilm cells of *G. vaginalis*, respectively. Furthermore, citral was effective in preventing the survival of *G. mellonella* larvae from the exposure of *G. vaginalis* by reducing the *in vivo* bacterial load. This was confirmed further by histopathological examination, which unequivocally shows that citral protects *G. mellonella* tissues from *G. vaginalis* infection. Further, the attempts made to

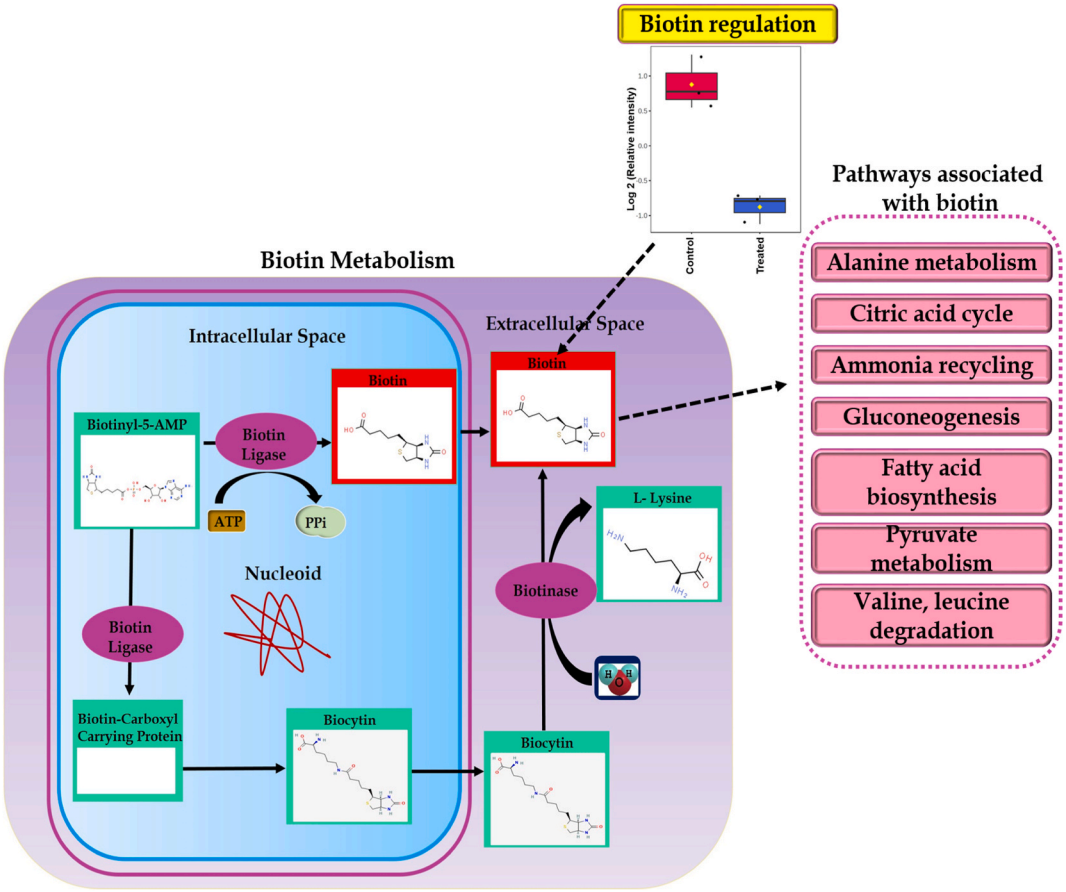

Fig. 11. The overview of most impacted biotin metabolism and its associated metabolic pathways. The metabolite linked with biotin metabolism was highlighted in red color.

understand the molecular action of citral *in vitro* (via PI staining and an intracellular protein leakage experiment) signify the possible degradation of *G. vaginalis*' membrane integrity. The untargeted metabolomic analysis using LC-MS uncovered the influence of citral on multiple fundamental metabolic pathways (*viz.*, polyamine, amino acid, fatty acid, and nucleotide) especially influencing biotin metabolism, which are essentially required for the basic metabolic viability of BV-causing *G. vaginalis* cells.

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Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

CRediT authorship contribution statement

Ravi Jothi: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Arumugam Kamaladevi:** Software, Methodology. **Pandiyan Muthuramalingam:** Software, Methodology. **Nambiraman Malligarjunan:** Software. **Shunmugiah Karutha Pandian:** Writing – review & editing, Formal analysis. **Shanmugaraj Gowrishankar:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization.

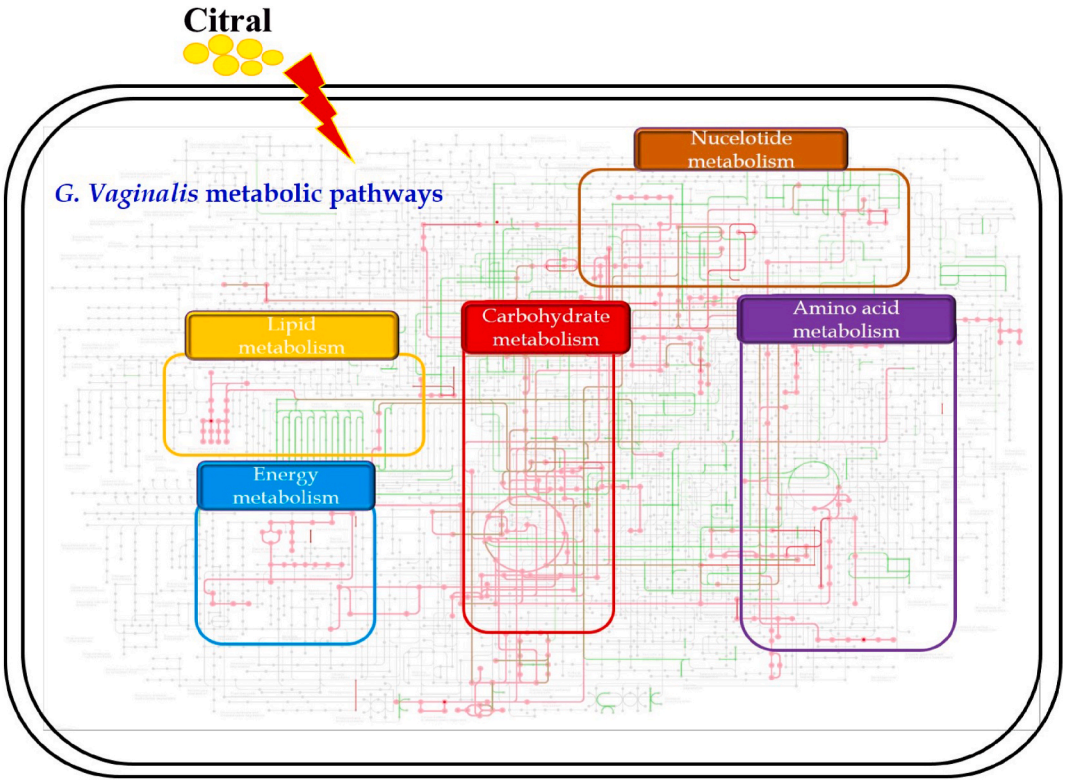

Fig. 12. Overall impact of citral treatment on the metabolic pathway of *G. vaginalis* (*G. vaginalis* KEGG database).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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