Verma et al | Article 9418
J Pure Appl Microbiol. 2025;19(1):594-600. doi: 10.22207/JPAM.19.1.49
Received: 30 March 2024 | Accepted: 18 November 2024
Published Online: 03 March 2025

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Exploring the Antimicrobial Potential of Hesperetin: A Study on Clinically Relevant Bacterial and Fungal Pathogens

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Abstract

Hesperetin, a natural flavonoid, has been investigated for its potential therapeutic properties. In this study, we delve into its antimicrobial and antiviral activities against clinically relevant bacterial and fungal strains. Our investigation unveiled substantial antimicrobial and antioxidant properties of hesperetin against a diverse array of pathogens, encompassing both Gram-positive as well as Gram-negative bacteria. Furthermore, notable antifungal activities were observed, particularly against resistant fungal strains. The findings from our study underscore the potential of hesperetin as a promising candidate for the development of broad-spectrum antimicrobial agents. Overall, hesperetin exhibits versatility with implications for combating infectious diseases. These insights pave the way for further exploration of hesperetin's therapeutic applications and its potential utility in addressing the challenges posed by antimicrobial resistance.

Keywords: Hesperetin, Antimicrobial, Gram-negative, Gram-positive, Antifungal, Antioxidant

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Citation: Verma A, Bakshi J, Kumar G, et al. Exploring the Antimicrobial Potential of Hesperetin: A Study on Clinically Relevant Bacterial and Fungal Pathogens. *J Pure Appl Microbiol.* 2025;19(1):594-600. doi: 10.22207/JPAM.19.1.49

INTRODUCTION

Hesperetin, a flavonoid naturally found in abundance in citrus fruits, has attracted substantial interest lately due to its promising therapeutic effects. Flavonoids, including hesperetin, are acknowledged for their wide-ranging biological activities, encompassing anti-oxidant, anti-inflammatory, and anti-microbial properties.[1,2] Among these, the antimicrobial potential of hesperetin has emerged as an area of particular interest, given the growing concern surrounding antimicrobial resistance and the need for novel therapeutic agents.[3]

In this study, we aim to explore the antimicrobial and antiviral activities of hesperetin against a spectrum of clinically relevant bacterial and fungal strains. By investigating its efficacy against a diverse array of pathogens, encompassing both Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria, as well as resistant fungal strains, we seek to elucidate hesperetin's potential as a broad-spectrum antimicrobial agent.

E. coli, a Gram-negative bacterium commonly inhabiting the lower intestine of warm-blooded organisms, can include pathogenic strains causing gastrointestinal illnesses, urinary tract infections, and severe conditions like septicaemia and meningitis.[4] These pathogenic strains produce toxins damaging the intestinal lining, leading to symptoms such as diarrhoea, abdominal pain, and vomiting, emphasizing the need to combat *E. coli* infections for public health.

B. subtilis, a Gram-positive bacterium found in soil and animal gastrointestinal tracts, is generally non-pathogenic to humans. However, certain strains can cause opportunistic infections, especially in immunocompromised individuals, potentially leading to bacteraemia, pneumonia, or wound infections.[5] Despite being uncommon, these infections underscore the importance of understanding compounds like hesperetin's antimicrobial activity against *B. subtilis*.

Additionally, our study delves into hesperetin's antifungal properties against *Aspergillus fumigatus*. *A. fumigatus*, a filamentous fungus prevalent in soil and decaying organic matter, is both a vital decomposer in ecosystems and an opportunistic human pathogen causing a spectrum of respiratory infections known as aspergillosis.[6] In immunocompromised individuals, invasive aspergillosis can arise, characterized by severe pneumonia and disseminated fungal spread.[7] Non-invasive forms like allergic fungal sinusitis (AFS) and allergic broncho-pulmonary aspergillosis (ABPA) affect those with underlying lung conditions or allergies. Given the morbidity and mortality associated with invasive aspergillosis and limited treatment options, exploring compounds like hesperetin for antifungal activity against *A. fumigatus* offers promise in developing novel therapeutics for this clinically significant fungal pathogen.

Previous research has indicated the antimicrobial properties of various flavonoids, including hesperetin, against a range of bacterial and fungal species.[3,8] However, studies elucidating the antimicrobial and antiviral activities of hesperetin against clinically relevant pathogens are limited. Therefore, our study seeks to address this gap in the existing literature by offering valuable insights into the therapeutic capabilities of hesperetin in combatting infectious diseases and tackling the hurdles presented by antimicrobial resistance.

MATERIALS AND METHODS

Materials

Reagents

Hesperetin was purchased from Cayman chemical (Ann Arbor, MI, USA). DPPH (1,1-Diphenyl-2-picrylhydrazyl) and diclofenac sodium were supplied by Sigma Aldrich, (Bangalore, India). Sodium dihydrogen phosphate (NaH_2PO_4) and di-sodium hydrogen phosphate (Na_2HPO_4) were purchased from CDH Labs. (India). All of the procured reagents were of analytical grade.

Test cultures

The bacterial strains utilized for the anti-microbial assays, namely *Escherichia coli* and *Bacillus subtilis* were sourced from the National Collection of Dairy Cultures (NCDC), India. The fungal strain *Aspergillus fumigatus*, was sourced from the Microbial Type Culture Collection (MTCC) in Chandigarh, India. To ensure viability, these test microorganisms were preserved on agar slants, at a temperature of 4 °C. Prior to experimentation,

they were sub-cultured in their respective broth media.

Methods

Antibacterial activity

To assess the antibacterial activity, the agar well-diffusion method was utilized as mentioned previously.[9] Briefly, the bacterial stock cultures were sub-cultured using nutrient agar media. After 24 hours the bacterial cultures were transferred in a freshly prepared broth media. Using the McFarland scale, the bacterial population were standardized to 1 x 10^8 CFU/mL.[10] Thereafter, under aseptic conditions, the nutrient agar media was poured into petri dishes.

Subsequently, 100 µL of broth cultures were evenly spreaded on agar plates using an L-shaped spreader and allowed to settle. After 20-minute interval, two wells were punctured using a microtip into the solidified agar medium. Subsequently, two test samples (hesperetin and blank) were added to the respective wells, followed by incubation at 37 °C for 24 hours. After the incubation period, the growth inhibition zone was measured. Unless otherwise stated all of the experiments were conducted in triplicates.

In vitro antioxidant activity

The anti-radical activity was assessed through the radical scavenging activity of the 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), with ascorbic acid serving as the standard.[11] First, a fresh 0.2 mM DPPH solution having concentration of 78 µg/mL in 95% ethanol was prepared. Subsequently, 100 µl of test samples were mixed with an equal volume of the freshly prepared DPPH solution. The resulting solution was vigorously agitated and then left to incubate in darkness for 30 minutes. The absorbance of the test samples was measured at 517 nm using 95% ethanol as a reference, employing an ELISA reader (Synergy™ Multi-detection Plate Reader, Biotek Instruments Inc, USA). The measurements were performed in triplicates. Higher free radical scavenging capacity was indicative of lower absorbance values. The percentage inhibition of the DPPH radicals by the samples was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where,

$A_{control}$ = DPPH radical + ethanol

A_{sample} = DPPH radical + samples/standard

Antifungal susceptibility testing

Conidial spores of *Aspergillus fumigatus* (ATCC) were collected in phosphate-buffered saline supplemented with 0.05% Tween-20. Subsequently, the conidial suspensions were adjusted to a concentration of 1 x 10^4 conidia/mL in potato dextrose broth. The conidial cultures were harvested by centrifugation and dissolved in PBS, with a total cell concentration of 1 x 10^4 cells/mL.

Antifungal susceptibility testing of hesperetin was conducted to determine the Minimum Inhibitory Concentration (MIC) using the CLSI M38-A2 broth micro-dilution method in a 96-well polystyrene plate. The experiments were performed in triplicate for each strain. Two-fold dilutions of hesperetin (starting concentration: 5 mg/mL) were prepared in a 96-well microplate to achieve the final concentrations ranging from 5 to 0.0097 mg/mL.

Each well, except for the negative control, received 100 µL of conidial suspension. The plates were then incubated for 4 days at 28 ± 2 °C. The minimum inhibitory concentration (MIC) of hesperetin was identified as the lowest concentration at which no visible growth was detected in comparison to the control group devoid of the drug.[12]

Statistical analysis

The statistical analysis was conducted by utilizing GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). Statistical differences among the samples were estimated by two-tailed student's t-test. For p values < 0.05, the differences were identified as significant.

RESULTS

We initiated our investigation by examining the antimicrobial properties of hesperetin. Following established protocol,[13] we

cultivated both gram-positive (*Bacillus subtilis*) and gram-negative (*Escherichia coli*) bacterial strains on LB agar-coated Petri dishes, a standard medium conducive to bacterial growth. These dishes were then subjected to an overnight incubation period at 37 °C, allowing for the robust proliferation of bacterial colonies. Subsequently, two wells were carefully punctured into each culture dish to facilitate the application of hesperetin solution or a blank control.

Our experimental design entailed the utilization of hesperetin at a concentration of 20 mg/mL, chosen based on our previous study highlighting its efficacy as an antiviral agent against poxviruses.[14] As a comparative reference, we employed dimethyl sulfoxide (DMSO) as the control or blank. Following the application of hesperetin and the blank control, the culture dishes underwent an additional incubation period, allowing for the interaction between the test substances and the bacterial cultures. Upon completion of the incubation period, we assessed the extent of bacterial growth inhibition induced by hesperetin (Figure). The measurement of the zone of growth inhibition served as a quantitative indicator of the compound's antimicrobial efficacy against the tested bacterial strains (Table 1). Remarkably, our results revealed a

Table 1. Zone of inhibition activity of hesperetin and blank (DMSO)

Samples	Diameter of zone of inhibition (mm)	
	E. coli	*B. subtilis*
Hesperetin	3 mm ± 0.4	3.5 mm ± 0.7
Blank (DMSO)	0 mm	0 mm

Values are expressed as the mean ± SD of three replicates

Table 2. DPPH radical scavenging activity of hesperetin, vehicle control (DMSO) and ascorbic acid (positive control)

Samples	Inhibition (%)
Hesperetin	61.93 ± 0.86
Ascorbic acid (positive control)	84.68 ± 0.33
Blank (DMSO)	0.3 ± 0.02

Values are expressed as the mean ± SD of three replicates

Table 3. Minimum Inhibitory Concentration (MIC) of hesperetin and vehicle control (DMSO) against *A. fumigatus* growth

Samples	Minimum inhibitory concen. (MIC)
Hesperetin	0.625 mg/mL
Blank (DMSO)	none

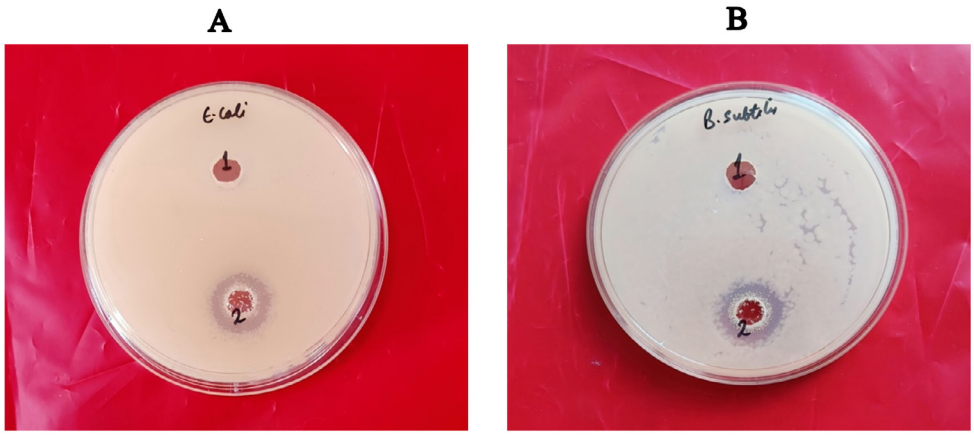

Figure. Antibacterial activity of hesperetin against (A) *E. coli* and (B) *B. subtilis*. Indicated strains were cultured on LB agar plates in triplicates. Holes were punched using biopsy puncher and blank (DMSO) or hesperetin 20 mg/ml were applied followed by overnight incubation. The zone-of-inhibition was calculated next day

substantial inhibition of bacterial growth across both of the tested strains, underscoring the potent antimicrobial activity of hesperetin.

DPPH is widely recognized in antioxidant research for its reliability and versatility as a radical species used to evaluate antioxidant capacity. The mechanism involves antioxidants donating hydrogen atoms to DPPH radicals, resulting in the formation of stable DPPH-H molecules.[15] In our study, we employed DPPH to assess the reducing power of samples, utilizing the color change from purple to yellow as an indicator of DPPH radical reduction, while the control exhibited no discernible color change.

Our results revealed the significant reducing activity of hesperetin, as evidenced by the observed reduction in DPPH activity (Table 2). Ascorbic acid, utilized as a positive control, exhibited the highest percentage of DPPH inhibition at 84.68 ± 0.33%. Notably, hesperetin demonstrated a substantial reduction in DPPH activity, with a percentage inhibition of 61.93 ± 0.86%, compared to the positive control. Conversely, the black (DMSO) control showed no significant reduction in DPPH activity, further corroborating the specificity of the observed antioxidant effects. The lack of DPPH reduction in the control underscores the importance of the active compound, hesperetin, in eliciting the observed antioxidant response (Table 2). These findings underscore the potent antioxidant activity of hesperetin, positioning it as a promising candidate for further exploration in the development of novel antioxidant therapies.

In addition to assessing its antimicrobial properties, we also explored the antifungal efficacy of hesperetin against clinically relevant strains of *Aspergillus fumigatus*. To conduct this evaluation, we prepared a two-fold dilution series of hesperetin, initiating from a concentration of 5 mg/mL. These dilutions were then incubated with the cultured suspensions of the fungal strains for a duration of 4 days, maintaining the temperature at 28 ± 2 °C to simulate optimal growth conditions. Following the incubation period, we observed the plates to visually determine the Minimum Inhibitory Concentration (MIC) of hesperetin. This critical parameter signifies the lowest concentration at which no visible growth of the fungal pathogens was observed. Our findings, summarized in Table 3, revealed compelling results regarding the antifungal activity of hesperetin.

Remarkably, hesperetin demonstrated potent inhibitory effects against both the hyphal growth of the pathogens and the germination of conidial spores. Notably, the MIC value of hesperetin was determined to be 0.625 mg/mL for *Aspergillus fumigatus* strain. This indicates that hesperetin effectively inhibited 50% of the growth of these fungal pathogen under the experimental conditions employed. These results highlight the promising potential of hesperetin as a therapeutic agent against fungal infections, underscoring its relevance in combating clinically significant pathogens. Further research endeavors could delve deeper into elucidating the precise mechanisms underlying hesperetin's antifungal activity, paving the way for its application in clinical settings as an alternative or adjunctive treatment option for fungal infections.

DISCUSSION

Our study demonstrates the multifaceted therapeutic potential of hesperetin, encompassing not only its antimicrobial activity but also its antioxidant properties. In addition to demonstrating significant antimicrobial efficacy against both bacterial and fungal pathogens, our investigation revealed notable antioxidant activity exhibited by hesperetin.

Antioxidants are pivotal in counteracting detrimental free radicals and reactive oxygen species (ROS) within the body, thus alleviating oxidative stress and diminishing the likelihood of cellular harm and disease.[16] The antioxidant properties of hesperetin stem from its ability to scavenge free radicals and inhibit oxidative processes, thereby protecting cells and tissues from oxidative damage.[17]

In our experiments, hesperetin exhibited potent antioxidant activity, as evidenced by its ability to reduce oxidative stress markers and enhance antioxidant enzyme activity *in vitro*. These findings are also consistent with the previous research demonstrating the antioxidant potential of hesperetin in various cellular and animal models.[18,19] Hesperetin's antioxidant activity may be attributed to its chemical structure,

which enables it to donate hydrogen atoms or electrons to free radicals, thereby neutralizing their reactivity and preventing cellular damage.

The dual action of hesperetin as both an antimicrobial agent and an antioxidant holds promising implications for its therapeutic use in combating infectious diseases and oxidative stress-related conditions. By targeting both microbial pathogens and oxidative stress pathways, hesperetin may offer a comprehensive approach to disease prevention and treatment.

Furthermore, the synergistic effects of hesperetin's antimicrobial and antioxidant properties warrant further investigation, particularly in the context of infectious diseases characterized by oxidative stress-induced tissue damage. Future studies exploring the mechanistic basis of hesperetin's dual activity and its potential synergism with conventional antimicrobial agents are warranted to elucidate its therapeutic potential fully.

In addition to its antimicrobial and antioxidant activities, we also investigated the antifungal efficacy of hesperetin against clinically relevant strain of *Aspergillus fumigatus*. Our results showed potent inhibitory effects of hesperetin against both hyphal growth and conidial germination of the fungal pathogens. These findings are also consistent with previous studies demonstrating the antifungal activity of hesperetin against various fungal strains.[20,21] The low MIC values of hesperetin further underscore its efficacy as an antifungal agent, highlighting its potential therapeutic utility in combating fungal infections.

Overall, the findings from our study, combined with previous literature, suggest that hesperetin possesses multifaceted pharmacological properties, including antimicrobial, antioxidant, and antifungal activities. These observations hold promising implications for the development of hesperetin-based therapeutic interventions for the management of microbial infections, oxidative stress-related disorders, and fungal diseases. Further research endeavours are warranted to elucidate the underlying mechanisms-of-action of hesperetin and to evaluate its efficacy in preclinical and clinical settings for the treatment of various pathological conditions.

CONCLUSION

This study demonstrates the multifaceted therapeutic potential of hesperetin, establishing its efficacy as an antimicrobial, antifungal, and antioxidant agent. Our findings revealed significant antimicrobial activity against both Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacterial pathogens, with zones of inhibition measuring 3.5 mm ± 0.7 and 3 mm ± 0.4, respectively. The potent antioxidant properties of hesperetin were confirmed through DPPH radical scavenging activity (61.93 ± 0.86% inhibition), while its substantial antifungal efficacy against *Aspergillus fumigatus* was demonstrated with a minimum inhibitory concentration of 0.625 mg/mL. These results collectively position hesperetin as a promising candidate for developing novel therapeutic interventions against diverse pathogens, while simultaneously addressing oxidative stress-related conditions through its robust antioxidant activity.

ACKNOWLEDGMENTS

The authors express their sincere gratitude to the Department of Bio and Nano Technology and the National Centre for Veterinary Type Cultures, NRCE, Hisar, for their invaluable support and seamless facilitation of this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

SK and SB conceived and designed the study. HK, GC and JB assisted in experimental design and execution. AV performed the experiments. RV, NK, SB and SK analysed the data. AV wrote the manuscript. RV, NK, SB and SK reviewed the manuscript. All authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

REFERENCES

1. Abrahao JS, Drumond BP, de Souza Trindade G, et al. Rapid detection of *Orthopoxvirus* by semi-nested PCR directly from clinical specimens: A useful alternative for routine laboratories. *J Med Virol.* 2010;82(4):692-699. doi: 10.1002/jmv.21617
2. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr.* 2004;79(5):727-747. doi: 10.1093/ajcn/79.5.727
3. Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. *Int J Antimicrob Agents.* 2005;26(5):343-356. doi: 10.1016/j.ijantimicag.2005.09.002
4. Russo TA, Johnson JR. Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. *Microb infect.* 2003;5(5):449-456. doi: 10.1016/S1286-4579(03)00049-2
5. Farrar WE, Reboli AC. The genus *Bacillus*-medical. *The Prokaryotes.* 2006;6:609-630. doi: 10.1007/0-387-30744-3_18
6. Hohl TM, Feldmesser M. *Aspergillus fumigatus*: principles of pathogenesis and host defense. *Eukaryotic Cell.* 2007;6(11):1953-1963. doi: 10.1128/EC.00274-07
7. Margalit A, Carolan JC, Kavanagh K. Bacterial interactions with *Aspergillus fumigatus* in the immunocompromised lung. *Microorganisms.* 2021;9(2):435. doi: 10.3390/microorganisms9020435
8. Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr.* 2001;74(4):418-425. doi: 10.1093/ajcn/74.4.418
9. Jain P, Nimbrana S, Kalia G. Antimicrobial activity and phytochemical analysis of Eucalyptus tereticornis bark and leaf methanolic extracts. *Int J Pharm Sci Rev Res.* 2010;4(2):126-128.
10. Andrews JM. BSAC standardized disc susceptibility testing method (version 8). *J Antimicrob Chemother.* 2009;64(3):454-489. doi: 10.1093/jac/dkp244
11. Cheng Z, Moore J, Yu L. High-Throughput Relative DPPH Radical Scavenging Capacity Assay. *J Agric Food Chem.* 2006;54(20):7429-7436. doi: 10.1021/jf0611668
12. Sen P, Vijay M, Kamboj H, Gupta L, Shankar J, Vijayaraghavan P. *cyp51A* mutations, protein modeling, and efflux pump gene expression reveals multifactorial complexity towards understanding *Aspergillus* section *Nigri* azole resistance mechanism. *Sci Rep.* 2024;14(1):6156. doi: 10.1038/s41598-024-55237-9
13. Bakshi J, Mehra M, Grewal S, Dhingra D, Kumari S. Synthesis, characterization and evaluation of *in vitro* antimicrobial and anti-diabetic activity of berberine encapsulated in guar-acacia gum nanocomplexes. *J Bioact Compat Polym.* 2022/07/01 2022;37(4):233-251. doi: 10.1177/08839115221106700
14. Verma A, Dedar RK, Kumar R, et al. Hesperetin blocks poxvirus replication with a low tendency to select for drug-resistant viral variants. *J Med Virol.* 2024;96(4):e29555. doi: 10.1002/jmv.29555
15. Sridhar K, Charles AL. *In vitro* antioxidant activity of Kyoho grape extracts in DPPH and ABTS assays: Estimation methods for EC_{50} using advanced statistical programs. *Food Chem*. 2019;275:41-49. doi: 10.1016/j.foodchem.2018.09.040
16. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. *Int J Biomed Sci.* 2008;4(2):89-96. doi: 10.59566/IJBS.2008.4089
17. Ganesan A, Mahesh A, Sundararaj JP, Mani K, Ponnusamy P. Antihyperglycemic and antioxidant activity of various fraction of *Parmotrema hababianum* in streptozotocin-induced diabetic rat. *Bangladesh J Pharmacol.* 2016;11(4):935-939. doi: 10.3329/bjp.v11i4.27658
18. Samie A, Sedaghat R, Baluchnejadmojarad T, Roghani M. Hesperetin, a citrus flavonoid, attenuates testicular damage in diabetic rats via inhibition of oxidative stress, inflammation, and apoptosis. *Life Sci.* 2018;210:132-139. doi: 10.1016/j.lfs.2018.08.074
19. Sykula A, Cheshchevik VT, Cheshchevik NG, Dzeikala A, Lodyga-Chruscinska E. *In vitro* study of hesperetin Schiff bases antioxidant activity on rat liver mitochondria. *Biotechnol Food Sci.* 2018;82(2):151-159.
20. Hung Y, Verbeke W, de Kok TM. Stakeholder and consumer reactions towards innovative processed meat products: Insights from a qualitative study about nitrite reduction and phytochemical addition. *Food Control.* 2016;60:690-698. doi: 10.1016/j.foodcont.2015.09.002
21. Gowtham R, Fysal YMA, Ezhilarasan D, Sambantham S, Anandan B. *In vitro* Antifungal Effects of Hesperetin and Silibinin. *Pharmacogn J*. 2018;10(4):789-792. doi: 10.5530/pj.2018.4.133