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RESEARCH ARTICLE

Investigation of Anti-Mycobacterial Activity of Orientin and Vitexin on the Six *Mycobacterium tuberculosis* Strains

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ABSTRACT

Objective: Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*, which causes disease in all organs, 80% of which are in the lungs, and can sometimes spread to other organs. It can lead to death in untreated or inadequately treated patients. Treatment of tuberculosis is very difficult due to the resistance of mycobacteria to many chemicals and disinfectants, antibiotics, and chemotherapeutics, especially in immunocompromised patients (HIV, Human Immunodeficiency Virus); this disease is very common. Therefore, in recent years, the search for new drugs to be used to treat tuberculosis has increased worldwide. We aim to determine the effect of orientin and vitexin on *M. tuberculosis* strains.

Materials and Methods: In this study, the effects of orientin and vitexin against *M. tuberculosis* standard strains (*M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv) and six-clinical *M. tuberculosis* strains. The inoculum was prepared using a positive BACTEC "Mycobacteria Growth Indicator Tube" containing 7H9 Broth. Microplate Presto Blue Method and rifampicin were used as standard antibiotics in the anti-mycobacterial assay.

Results: Orientin and vitexin showed a mycobactericidal effect on tuberculosis strains depending on the concentration. Orientin and vitexin have not been tested on current clinical strains of *M. tuberculosis* before. In this respect, it is the first report describing the anti-mycobacterial activity of both orientin and vitexin.

Conclusion: These results indicate that orientin and vitexin may be helpful for further investigations into their role in inhibiting *M. tuberculosis*. They have a possibility of new anti-mycobacterial drug candidates in the near future.

Keywords: *Mycobacterium tuberculosis*, orientin, vitexin, anti-mycobacterial activity, MIC, MBC.

INTRODUCTION

Tuberculosis (TB) is a chronic bacterial disease caused by a bacterium called *Mycobacterium tuberculosis* (MT), which can primarily attack the lungs and affect other organs. The bacteria that cause tuberculosis are spread from one person to another through tiny droplets released into the air through coughing and sneezing. TB is one of the top 10 causes of death worldwide. In 2016, 10.4 million people contracted the disease and 1.7 million died from the disease (including 0.4 million people with HIV). Over 95% of TB deaths occur in low- and middle-income countries.[1] In 2019, there were 1.2 million TB deaths among HIV-negative people and an additional 209,000 TB deaths among HIV-positive people. There has been an increase in tuberculosis deaths in poor countries where access to tuberculosis diagnosis and treatment has decreased.[2] In 2021, the burden of drug-resistant TB was estimated to increase to 450,000 new cases of rifampicin-resistant-TB.[3]

Resistance to rifampicin (RR-TB) burden, 450,000 new cases of rifampicin resistance were detected between 2020 and 2021. Especially, between 2019 and 2020, there was a decrease in the number of people treated for RR-TB[4] and multidrug-resistant TB (MDR-TB) due to the COVID-19 outbreak.[5] Nowadays, drug-resistant TB remains a public health threat. RR-TB, the most effective first-line drug, is a cause for concern. Resistance to rifampicin and isoniazid is defined as MDR-TB. Both RR-TB and MDR-TB require treatment with second-line drugs. Globally, the estimated number of people who develop MDR-TB or RR-TB each year was relatively stable between 2015 and 2020 but increased in 2021. According to The World Health Organization (WHO) 2022 Global Tuberculosis Report, it is stated that the reason for this increase is the negative impact of the COVID-19 pandemic on TB detection. Added to this is the decline in global spending on essential TB services, and economic and financial barriers to access to health care to diagnose and treat TB.[1]

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Orientin ($C_{21}H_{20}O_{11}$) is a water-soluble flavonoid synonymously known as -β-D-Glucopyranosyl-3,4,5,7 -tetrahydroxyflavone, Luteolin 8-C-β-D-glucopyranoside, Luteolin-8-glucoside or Lutexin (Figure 1A). Its molecular formula is $C_{21}H_{20}O_{11}$ and its molecular weight is 448.3769 g/mol.[6] Vitexin is a water-soluble flavonoid (Figure 1B). Its molecular formula is $C_{21}H_{20}O_{10}$ and its molecular weight is 432.381 g/mol.[7]

Orientin, is a flavonoid component, has been isolated from many medicinal plants. Among them, *Ocimum sanctum*, *Trollius chinensis, Phyllostachys pubescens*,[8] and *Passiflora incarnate*.[7] The properties of orientin have only just begun to be studied. Li et al.[9] investigated the effects of orientin on cardiac tissue remodelling after myocardial infarction and determined that orientin supplementation reduced oxidative stress in cardiac tissue and cardiomyocytes exposed to hypoxia. They also reported that orientin treatment increased hypoxia-induced neonatal rat cardiomyocyte apoptosis and cell viability in animal experiments. Endothelial nitric oxide synthase (eNOS signalling) regulates blood pressure via vascular smooth muscle contraction[10] and blood vessel vasodilation.[11] It was suggest that orientin is a promising neuroprotective agent suitable for the treatment of neuropathic pain.[12,13] Using Western blot analysis in their research, they showed that the Toll-like receptor mediates inhibition of the nuclear factor kappa-B signaling pathway. Orientin has been observed to protect heart and cardiomyocyte damage by regulating autophagy.[14–16]

In addition, orientin was also shown to inhibit the expression of matrix metalloproteinase-9 and interleukin-8. Orientin inhibits migratory and invasive responses by suppressing metalloproteinase-9 and interleukin-8 expression. Kim et al.[17] suggested that orientin inhibits tumour invasion and is applicable as a possible therapeutic agent for the treatment of cancer metastasis. Orientin, a C-glycosyl dietary flavone abundantly found in Rooibos tea and passion fruit, has received great attention for its multiple pharmacological potentials. Thangaraj et al.[18] investigated the antiproliferative and anti-inflammatory effects of orientin in rats with 1,2-dimethyl hydrazine (DMH)-induced colorectal cancer. In this study, they showed that orientin inhibited the overexpression of inflammatory cytokines induced by 1,2-dimethyl hydrazine, thus revealing its antiproliferative and anti-inflammatory potentials.

Various plants have also been used to isolate orientin. Of these, *Trollius chinensis* is known as the "Golden Queen".[8,19] Regarding other biological activities, Yoo et al.[20] reported their anti-oxidant, anti-viral, anti-inflammatory activities to Wang et al.[21], and Xiao et al.[22] investigated their anti-glycation, anti-cancer and anti-thrombus activities.

Chen at al.[23] developed the ultrasonic circulatory extraction (UCE) approach for the effective removal of orientin and vitexin from flowers of *T. chinensis* and investigated some parameters that potentially affect the yield of orientin and vitexin.

The flowers of T. chinensis are a rich source of flavone-C-glycosides such as orientin and vitexin, and the most abundant bioactive flavonoid among flowers is orientin.[23,24] There are also various plants in which the orientin is determined. Arugula plant, *Eruca sativa* Mill, which is widely used to treat various diseases as a component of salads, as well as a folk remedy. Among the (*Brassicaceae*) flavonoids, orientin was found to be the main compound.[25]

Achillea species, one of the medicinal plants with many activities and used since ancient times, *Achillea nobilis* L. subsp. *neilreichii* orientin and vitexin were found in studies on ethyl acetate and ethanol extracts.[26]

Vitexin is, also known as apigenin flavone glycoside,[7] present in many plants and plant parts such as fruits mung beans trees and seeds, bamboo,[27] *Crataegus pinnatifida*,[28] pigeonpea leaves (*Cajanus cajan*),[29] and *Passiflora cristalina*.[30]

Some glycosylated flavonoids have a direct bond between the sugar and the anomeric carbon (O-C bond), while others, such as vitexin, have a sugar bond at C6 or C8 (C-C bond).[31] Vitexin and isovitexin are active ingredients in many traditional Chinese medicines. Vitexin (apigenin-8-C-glucoside) is receiving increasing attention due to its neuroprotective effects,[32] anti-inflammatory, antihypertensive,[30] anti-oxidant, anti-cancer,[29] and anti-tumor and anti-angiogenesis effects against cervical cancer cells.[33] Bhat et al.[34] reported that vitexin proved to be an effective inhibitor of hypoxia-inducible factor 1α (HIF-1α) in chondrocytes during osteoarthritis. New research suggests that vitexin may be potential alternative medicines or ancillary health products that can be used in various diseases.

In this study, we investigated the efficacy of orientin and vitexin against tuberculosis, which is one of the leading causes of death worldwide until the coronavirus (COVID-19) pandemic and is on top of human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) caused by a single infectious agent.

Kim et al.[35] studied seven flavonoids and showed that vitexin and orientin inhibited triglyceride accumulation the highest (approximately 40% and 33% at 100 μm respectively). They reported that other flavonoids (luteolin, chrysoeriol, cosmosin, apigenin, and luteolin-7-O-β-D-glucoside) showed lower levels of inhibition.

Drug-resistant TB remains a public health threat over the world. In this study, our aim is to investigate the effectiveness of orientin and vitexin against the tuberculosis agent *M. tuberculosis*, which is very difficult to treat due to its resistance to many chemicals, disinfectants, chemotherapeutics and antibiotics.

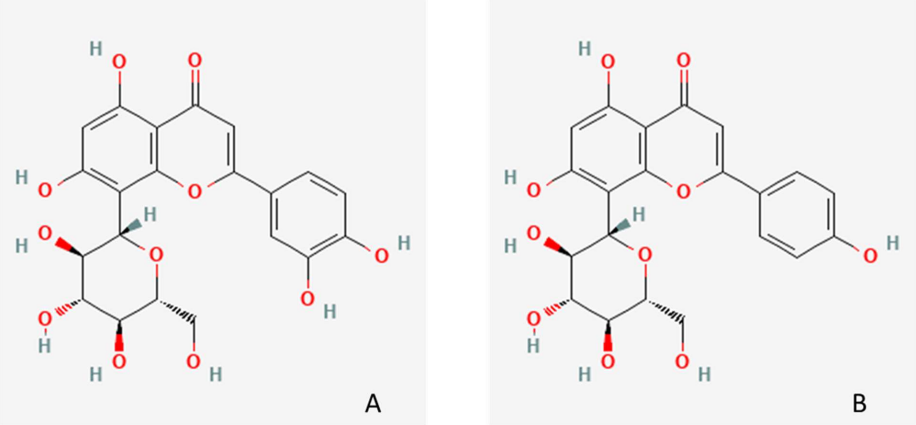

Figure 1. Chemical structure of orientin (A) and vitexin (B).

MATERIALS AND METHODS

Preparation of the Samples, Solutions, Microorganism and Inoculum Anti-Mycobacterial Assay

The compounds, orientin, (Sigma-Aldrich 55736) and vitexin (Sigma-Aldrich 49513), were obtained from Sigma-Aldrich. Ten mg were taken from the samples and dissolved in 0.5 ml dimethyl sulfoxide (DMSO). The stock solution concentration was 20 mg/mL. All stock solutions were stored in a deepfreeze at -20 ℃. To prepare 10 mL (1280 µg/mL concentration) solution, 0.64 mL of stock solution was taken and 9.36 mL of DMSO was added. Therefore, the final solution concentrations were 1280 µg/mL. The range of working solution concentrations in the wells was between 640-1.25 µg/mL. The extracts were tested against avirulent MT H37Ra (MT-Ra, ATCC 25177) and virulent MT H37 Rv (MT-Rv, ATCC 25618) from the American Type Culture Collection. Six other strains (PS-1 to PS-6) were obtained from the Balıkesir Chest Diseases Hospital tuberculosis laboratory. Anti-mycobacterial activity tests were performed in two series.

Aseptically added OADC (oleic acid, albumin, dextrose, and catalase-0.5 mL) and PANTA (polymyxin-B, amphotericin-B, nalidixic acid, trimethoprim, azlocillin-0.1 mL) antibiotic mixture into the MGIT (Mycobacteria Growth Indicator Tube-4 mL), containing modified MGIT tubes were incubated at 37 ℃. Inoculum made from a positive BACTEC MGIT tube was used one day after the tube became positive (Day1) and up to the fifth day (Day5). Day1 and Day2 positives were used directly for susceptibility testing, while Day3-Day5 positives were diluted 1:5 (1 ml positive broth into 4 ml sterile saline) and used for inoculum.[36–39] MGIT (4 mL), containing modified Middlebrook 7H9 Broth Base (MBB) was used to grow the strains at 37 ℃. Blood agar was used for each test to control the growth of suspicious bacteria other than MT. For this, the vials were tested daily, starting from the second day of incubation, using a MicroMGIT Fluorescent reader with long-wave UV light.

Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Determination of MIC for anti-mycobacterial assay, the microdilution method was achieved according to the CLSI, Susceptibility Testing of *Mycobacteria, Nocardia*, and Other Aerobic *Actinomycetes* guidelines.[40] The medium in MGIT tubes prepared as mentioned above was put into each well (100µL), and a sample (100 µL) at the concentration of 1280 µg/mL was added to the first well only. The volume of the first tube is 200 µL (sample solution and medium), while the others are 100 µL (only medium). 100 µL volume of the solution is transferred from the first well to the second well, by mixing three times with an automatic pipette. The volume of the first well is halved, but the concentration remains the same. In the second well, its volume doubled and its concentration was halved and this procedure included the 10^{th} well.

After the solution in the first well was mixed three times with an automatic pipette, the dilution was repeated from the first row to the 10^{th} row. The experiments also included positive and negative controls. Row 11 was positive and row 12 was the negative control. A 15 µL of MT suspension as inoculum was added to all wells except row 12. Then the tubes were incubated at 37 ℃. After the start day, the tubes were read daily. The day for positive was 8-12 days for MT. Results were evaluated using Presto Blue, a non-toxic, resazurin-based solution, that indicator which is a cell viability indicator. Metabolically active cells as pink and inhibited cells as blue were observed. For MBC determination, the inoculum was taken from the MIC wells and higher concentration wells and then added to wells containing fresh and sterile 7H9 medium. The plates were incubated at 37 ℃. Colour change in positive and negative control wells was

Table 1. The MIC and MBC values of orientin (µg/mL).

Anti-mycobacterial activity of orientin and vitexin (µg/mL)					Antibiotic (µg/mL)	
	Orientin		Vitexin		Rifampicin	
Microorganisms	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
MT-Ra	160	640	80	320	0.64	5.12
MT-Rv	320	640	80	320	0.32	2.56
PS-1	160	160	80	80	-	-
PS-2	160	160	80	160	-	-
PS-3	320	640	160	>640	-	-
PS-4	640	640	320	320	-	-
PS-5	320	320	160	320	-	-

MIC:Minimum Inhibition Concentration MBC:Minimum Bactericidal Concentration PS: Patient strain

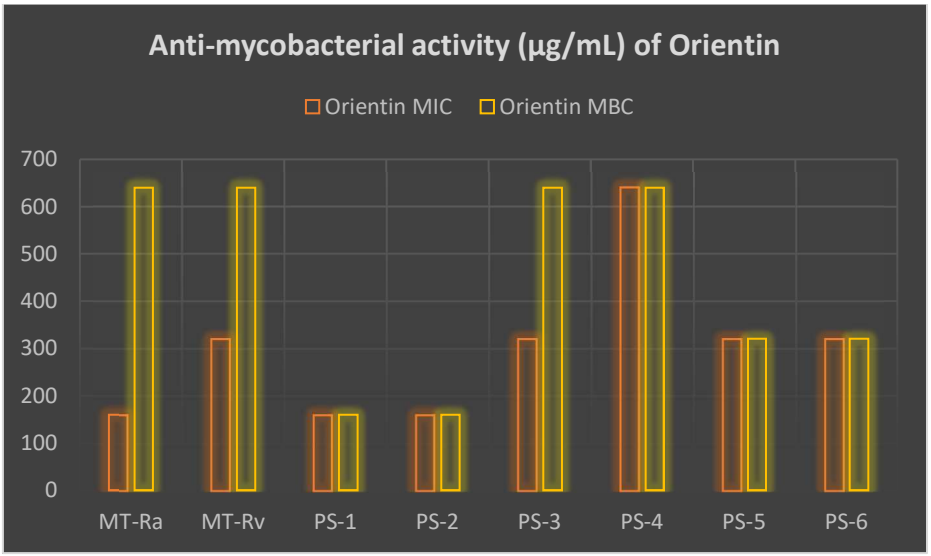

Figure 2. Anti-mycobacterial activity of orientin (µg/mL).

checked with Presto blue indicator. The lowest concentration without bacterial growth was accepted as MBC.

RESULTS

Orientin and vitexin stock solution and working solution (1280 µg/mL) were prepared. The concentration ranges from well-plate 1 to well 10 were adjusted as 640-1.25 µg/mL by serial dilution. Eight bacteria were used the determine the anti-mycobacterial activity of orientin and vitexin. Two of them were MT-Ra and MT-Rv standard bacteria, and the six clinical MT patient strains, (PS-1, PS-2, PS-3, PS-4, PS-5, and PS-6) were used as test organisms for MIC and MBC tests.

In our assays, the lowest MIC values determined for orientin were found against MT-Ra, PS-1, and PS-2 (MICs value were 160 µg/mL), and the MBCs were 640, 160, and 160 µg/mL, respectively. PS-1 and PS-2 showed the minimum MBC values. They have the same MIC and MBC values. On the other hand, the highest MIC and MBC value determined was PS-4 at 640 µg/mL. On the other hand, the highest MIC and MBC value determined PS-4 as 640 µg/mL. Depending on the concentration, orientin showed a mycobactericidal effect on tuberculosis strains (Table 1, Figure 2).

The lowest MIC values determined for vitexin were found against MT-Ra, MT-Rv, PS-1, and PS-2 (MIC values were 80 µg/mL). The MBCs were 320, 320, 80, and 160 µg/mL, respectively. MIC values obtained for vitexin were lower than that of orientin. The lowest MIC values of 80 µg/mL were observed in

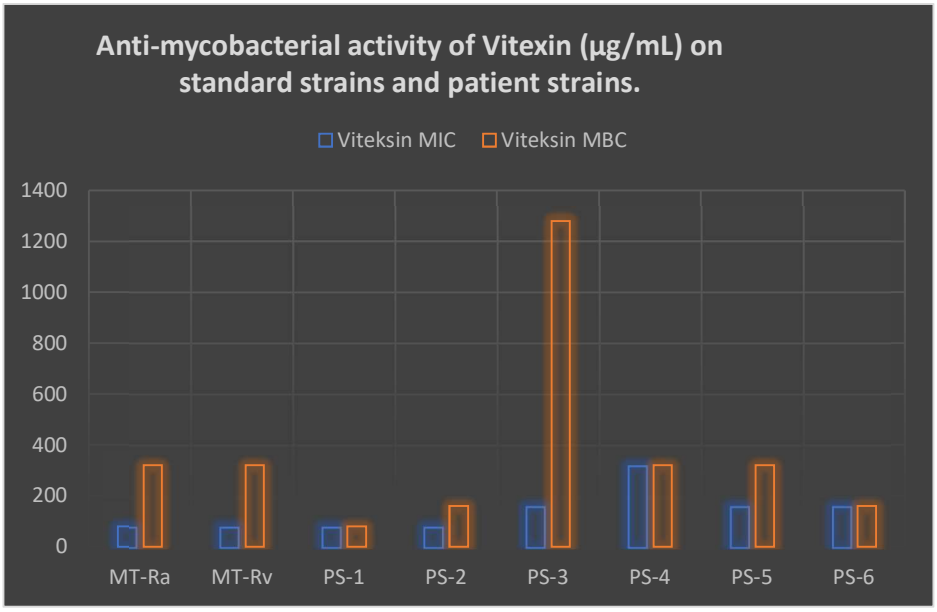

Figure 3. Anti-mycobacterial activity of vitexin (µg/mL).

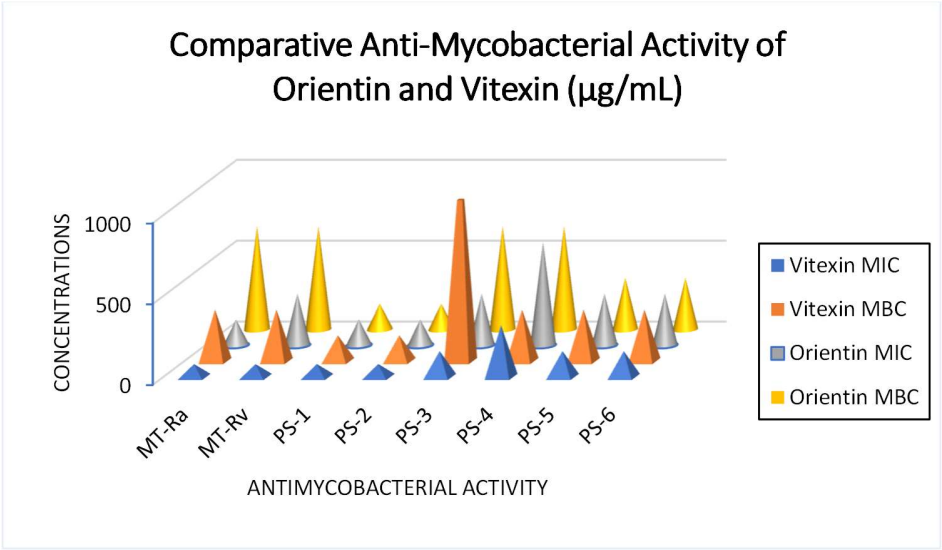

Figure 4. The comparative anti-mycobacterial activity of orientin and vitexin (µg/mL).

MT-Ra, MT-Rv, PS-1, and PS-2. While the MBC value for PS-1 was the same as the MIC value, the MBCs were 160 µg/mL for PS-2 and 320 µg/mL for MT-Ra and MT-Rv. Also, the highest MBC was found at PS-3 (MBC>640 µg/mL). The effects of vitexin on microorganisms are given in Table 1, Figure 3.

The comparative anti-mycobacterial activity of orientin and vitexin (µg/mL) is given in Figure 4. We found vitexin is more effective against Mycobacteria at lower concentrations than orientin.

DISCUSSION

There are no studies on the effects of orientin and vitexin on TB patient strains. Therefore, the antimycobacterial effect on patient strains is of great importance in terms of the potential drug substance. As a result of our research, we found that both orientin and vitexin (excluding PS-4, MBC>640 µg/mL) were highly effective against MT strains and showed mycobactericidal activity. When comparing the anti-mycobacterial activity (µg/mL) of orientin versus vitexin,

we determined that flavonoids vitexin was more effective at lower concentrations than orientin. Rifampicin, also recognized as rifampin, has a bactericidal effect on both extracellular and intracellular on Mycobacteria. We preferred to use rifampicin as the standard substance (antibiotic) because of its below-mentioned properties and because it is the most potent first-line anti-tuberculosis drug. It inhibits the RNA polymerase enzyme of Mycobacteria.[41] Rifampicin is a broad-spectrum semi-synthetic antibiotic obtained by fermentation of *Nocardia mediterranei*. It is an antimycobacterial agent that is effective at low concentrations against Mycobacteria.[42–43] Rifampicin stops bacterial growth by inhibiting RNA synthesis. The sensitivity of RNA polymerase is in good agreement with MIC values in Gram-positive bacteria. The higher MIC values observed in Gram-negative bacteria are due to less penetration of rifampicin into the outer membrane of these organisms.[44] The development of resistance is slower than in other bacteria. It has been shown to have the longest post-antibiotic effect.[45]

A study in the literature investigating the effect of orientin and isoorientin on macrophage cells was performed by Jesus et al.[44] In this study, the effects of orientin and isoorientin obtained in *Vitex polygamma* dichloromethane fraction on macrophage cells were examined. In their research, they investigated the antimycobacterial effect of orientin against the intracellular and extracellular growth of MT- H37Rv. In conclusion, they reported that it was able to reduce the growth of virulent MT-H37Rv and hypervirulent MT-M299. Besides, orientin presented a higher antimycobacterial activity. The antimycobacterial activity results of orientin on patient strains and standard strains in our study are in line with the results of Jesus et al.[46] They stated that the position of the C-glycosylation of the luteolin A-ring of orientin was effective in the action against *Mycobacterium*. Apart from Jesus et al.[46] there is no reported article on the antimycobacterial activity of orientin and vitexin in the literature. The effect of orientin and vitexin has not been tested on patient strains before. Therefore, our study is unique and fills the gap in this field.

Adamczak et al.[47] reported moderate antibacterial activities of orientin and vitexin against *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa* (MIC 500–1000 µg/mL). In this respect, MIC values and tested concentrations for the bacteria mentioned above are consistent with our results.

Song et al.[48] stated that the main antibacterial mechanisms of these flavonoids from *Trollius chinensis* Bunge due to the components (4'-methoxy-2"-O-(2-methylbutyryl) and 2-O-(3-methoxycaffeoyl) for vitexin and the component of 4'-methoxy-2"-O-(2-methylbutyryl) for orientin. They noted that the antibacterial mechanism of these components is through binding to DNA.[48]

Coumarins are also known to have moderate activity against *M. tuberculosis* and this activity is attributed to prenyl at the C-8 position.[49] However, the antimycobacterial activities of C-glycoside flavonoids (orientin and vitexin) on patient strains have not been studied.

In our study, the antimycobacterial activities of orientin and vitexin were tested on reference strains and patient strains. When the anti-mycobacterial activity of orientin and vitexin was compared, it was found that vitexin showed higher activity than orientin on MT strains. To the best of our knowledge, this study is the first report describing the anti-mycobacterial activity of orientin and vitexin against strains obtained from MT patients. However, more detailed studies are needed in areas such as pharmacology, toxicology, drug development, pharmacokinetics, pharmaceutical chemistry, drug release and clinic in order to use vitexin as a potential active drug substance. The fact that these above-mentioned stages have not yet been carried out creates a limitation in terms of demonstrating the effectiveness of orientin and vitexin.

CONCLUSION

Orientin and vitexin are bioactive compounds that can be isolated from medicinal plants and are promising flavonoids, which inhibit current strains of *M. tuberculosis*. Orientin and vitexin, rich sources of flavone-C-glycosides and the most abundant bioactive flavonoids among flowers, can potentially be promising compounds for further studies to treat MDR-TB. However, these studies require a lot of time, labour, and resources. Studies on some of their biological properties are reported. However, there is a need for a better understanding of pathways and mechanisms of action for vitexin and orientin to develop highly effective drugs with fewer side effects.

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