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Original Article

ANTIFUNGAL ACTIVITY OF LIPASE MODIFIED FLAVONOIDS FROM *CITRUS LIMETTA*

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ABSTRACT

Objective: The aim of the present study is to bring about enzymatic modification of flavonoids from *Citrus limetta* in order to increase their antifungal activity.

Methods: Methanolic extraction of flavonoids from citrus peels was carried out and their presence was confirmed by various tests. Crude extract of flavonoids was subjected to enzymatic modification by enzyme lipase in presence of acyl donor tributyrin in order to bring about esterification. Esterification was confirmed using IR spectroscopy and sodium hydroxide- phenolphthalein test. Antifungal activity of the treated sample and control sample were tested against *Candida albicans* and compared with fluconazole by Kirby Bauer disk diffusion method.

Results: Methanolic extract showed the presence of flavonoids on carrying out various test. While testing enzymatic modification, IR spectra did not show a significant difference between treated and control; however, enhanced esterification was confirmed by sodium hydroxide-phenolphthalein test. On carrying out antifungal activity, treated samples showed a 56.52% increase in zone of inhibition against *Canidida albicans* as compared to the control and was also found to be more in comparison to fluconazole.

Conclusion: The results signify that enzymatic modification increases the antifungal activity of flavonoids from *Citrus limetta*.

Keywords: *Citrus limetta*, Flavonoids, Lipase, Ester, Antifungal.

INTRODUCTION

Flavonoids are a large group of polyphenolic secondary plant metabolites known for their nutritional and pharmacological properties [1, 2]. Flavonoids are widely distributed in fruits, vegetables, cereals, cocoa, teas and wines. Citrus fruits like oranges, mandarins, grapefruits, lemons, bergamots, limes, accumulate substantial quantities of flavonoids in different organs during their development and therefore stand out as rich sources for the same [2,3]. Among these citrus fruits, *Citrus limetta,* commonly known as sweet lime in English and Mousambi in India, is rich in flavonoids such as hesperidine and naringin primarily found in the peel and inner membranous part of the fruit [4, 5,6].

These flavonoids are credited to have antibacterial, antifungal, antioxidant, antiviral, anticancer and analgesic properties [6]. However, complete therapeutic use of these flavonoids is restricted due to their low solubility and poor stability in polar and non-polar systems [7, 8].

To improve the bioactivity of these flavonoids, chemical or enzymatic structural modification is required [1, 8]. The presence of numerous reactive hydroxyl groups in flavonoids makes enzymatic modification a preferred route [1]. Enzymatic transformations are also cost effective due to their large availability, wide substrate spectrum, and no additional cofactor requirement [8]. To date enzymes like lipases, estarases, proteases, transferases, have been used as potent catalyst for flavonoid acylation [7].

Among these fungal, bacterial, and mammalian lipases have been widely used for biotransformation as they catalyse a wide range of reactions mainly esterification [9]. Esterification allows addition of acyl group in the molecule of flavonoid at the reactive hydroxyl group; which, may stabilize phenol function and increase compound lipophilicity hence enhancing the bioactive principles of these flavonoids [10].

Present investigation involves enzymatic modification of flavonoids from *Citrus limetta* by use of enzyme lipase in presence of an acyl donor. The increase in bioactive principles of these flavonoids was studied in terms of altered antifungal activity after enzymatic modification.

MATERIALS AND METHODS

Collection and processing of the plant material

Citrus limetta peels were collected and dried in the hot air oven at 60^{o}C for a period of 4 days. The dried plant material was powdered using a mechanical grinder and stored in airtight plastic bottles.

Flavonoid extraction from plant material

15 g of plant material was extracted in 75 ml of 80% methanol in a tightly capped conical flask. This mixture was briefly sonicated and maintained in a temperature controlled water bath at 50^{o}C for a period of 2-3 hours, and further refrigerated overnight. The methanolic layer was then filtered out and defatted using petroleum ether in the ratio of 10:1. The methanolic layer was filtered and concentrated in a pre weighed beaker to evaluate the percentage yield of the crude extract using the formula:

Extractive yield value= (Weight of concentrated extract / Weight of plant dried powder) ×100 [11] (Table. 1). The concentrated extract was reconstituted in 80% methanol to obtain a 50% crude extract of flavonoids.

Detection of flavonoids from the crude extract

Presence of flavonoids in the extract was detected using ultraviolet (UV) spectroscopy in the range of 200-400 nm. Ferric chloride test for identification of flavonoids was carried out by adding 1 ml of 0.1% ferric chloride to 0.1 ml of crude extract, color change was observed. Further, Shinoda test was performed, by adding drops of concentrated HCl to 0.5 ml of extract in the presence of magnesium, color change was observed.

Enzymatic modification of flavonoids

Enzyme lipase HIMEDIA® was procured and reconstituted in 0.1M PBS, pH 6.5. 1% of crude extract of flavonoids was subjected to the action on 100U of enzyme lipase in the presence of acyl donor tributyrin reconstituted in 2% polyvinyl alcohol. A ratio 1:5 of flavonoid: tributyrin was maintained. The resulting mixture was incubated at 50^{o}C. Aliquots were taken at 0 hour (control) and after 8 days (treated).

Detection of enzymatic modification

Infrared (IR) spectroscopy was carried out at Shri Chotabhai B Patel Research centre, Mumbai, for treated and control sample to detect function group change after enzymatic transformation. Esterification was determined by detecting the characteristic fragrance of esters. Further analysis of esterification was done using sodium hydroxide – phenolphthalein test wherein a few drops of 0.1N NaOH were added to the samples in presence of indicator phenolphthalein and maintained in a boiling water bath for 15 minutes. Color change was observed.

Antifungal activity

The antifungal activity of treated and control samples was tested against laboratory maintained strains of *Candida albicans* using Kirby-Bauer disk diffusion method by impregnating paper disks with 25 µl of each of the samples. The antifungal activity was also compared with 10 mcg fluconazole paper disks procured from HIMEDIA®. Appropriate controls were maintained.

RESULTS

In the crude methanolic extract obtained (Table. 1), the presence to flavonoids from *Citrus limetta* peels was detected using UV spectroscopy, which displayed a peak between 260-290 nm. The presence of flavonoids was further confirmed by Ferric chloride test and Shinoda test (Table. 2).

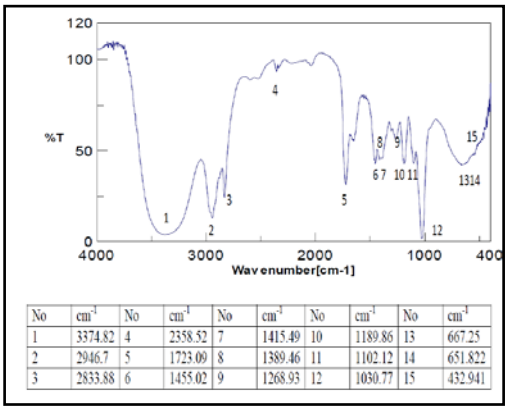
Enzymatic transformation was studied by IR spectroscopy, which revealed the presence of ester bonds at a frequency of 1723.09 cm^{-1} and 1725.98 cm^{-1} in the treated and control sample respectively (Fig.1, 2).

Table 1: Percentage yield of crude extract of flavonoids

Solvent	Weight of plant material	Volume of solvent	Weight of concentrated extract	% yield of crude extract
80% methanol	15 gm	75 ml	9.4 gm	62.66 %

Table 2: Phytochemical test to detect the presence of flavonoids

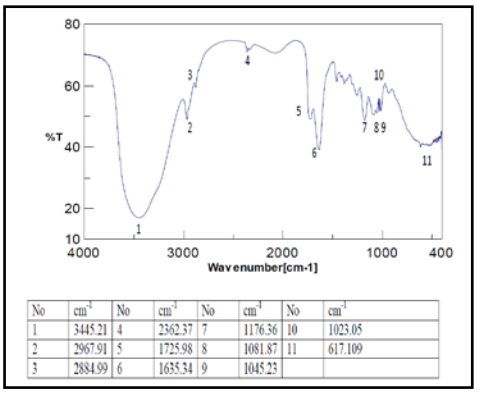
Phytochemical	Test	Observation	Result
Flavonoids	Ferric chloride	Pink red coloration	Flavonoids present
Flavonoids	Shinoda	Red coloration	Flavonoids present



No	cm^{-1}	No	cm^{-1}	No	cm^{-1}	No	cm^{-1}	No	cm^{-1}
1	3374.82	4	2358.52	7	1415.49	10	1189.86	13	667.25
2	2946.7	5	1723.09	8	1389.46	11	1102.12	14	651.822
3	2833.88	6	1455.02	9	1268.93	12	1030.77	15	432.941

Fig. 1: IR profile of the treated extract

On carrying out sodium hydroxide-phenolphthalein test, the color of the treated sample was significantly faint (light pink to colorless) as compared to the control sample, which appeared darker (Fig. 3). The treated sample was also confirmed to have enhanced fruity fragrance of esters.



No	cm^{-1}	No	cm^{-1}	No	cm^{-1}	No	cm^{-1}
1	3445.21	4	2362.37	7	1176.36	10	1023.05
2	2967.91	5	1725.98	8	1081.87	11	617.109
3	2884.99	6	1635.34	9	1045.23		

Fig. 2: IR profile of the control extract

Fig. 3: Difference in amount of ester present depending on intensity of color obtained

The antifungal activity of treated samples (Table 3) showed a 56.52% increase in the zone of inhibition against *Canidida albicans* as compared to the control. Also, the antifungal activity of the treated sample against *Canidida albicans* was found to be more in comparison to fluconazole (Fig. 4)

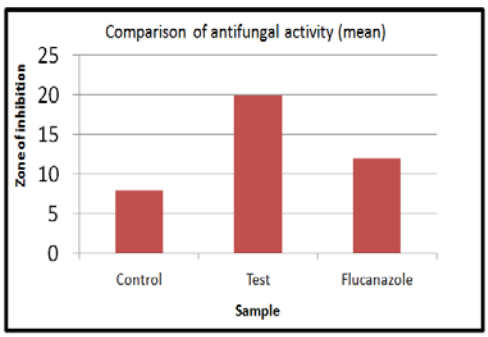


Fig. 4: Comparison of Antifungal activity of Test, Control and Fluconazole

DISCUSSION

Flavonoids from citrus peels were extracted using methanolic extraction, which is a widely used high yielding technique that gave us a crude extract of 62.66% [12]. *Citrus limetta* peels and inner membranous part of the fruit primarily contain flavonoids, hesperidine, and naringin, which are further classified into the group of flavonones [5, 13]. The characteristic UV spectra for these flavonone moieties lie between 270-295 nm [13]. On carrying out UV spectroscopy, our results display a peak between 260-290 nm, confirming the presence of flavonoids in the extract. The presence of flavonoids in the extract was further confirmed by a positive Ferric chloride and Shinoda test [14].

Flavonoids from *Citrus limetta* have pharmacological importance therefore improving their stability and solubility in hydrophilic and lipophilic systems is essential [7]. Enzymatic modification using enzyme lipase brings about esterification of flavonoids, which may increase their bioactivity; thus, making them therapeutically more useful. [7,8,10,15]. After carrying out enzymatic modification, IR spectroscopy was carried out to detect functional group change after treatment. IR spectroscopy revealed no significant change. However, Sodium hydroxide- phenolphthalein test showed a significant difference between the treated sample and the control sample.

The treated sample shows a much faint pink color than the control sample, this is due to the fact that ester bonds from the flavonoids will break down on boiling, thus increasing the acidity of the solution resulting in a color change of the indicator from dark pink to light pink.

The significant difference in color of the control and the treated sample may be indicative of enhanced esterification after enzymatic modification [16]. Esters have a characteristic pleasant fragrance which was more evident in the treated sample is another indicative of enhanced esterification [17, 18].

Table 3: Antifungal activtity of Test, Control, Fluconazole

Sample	Diameter of zone of inhibition (in mm)						
	1	**2**	**3**	**4**	**5**	**Mean**	**SD**
Citrus limetta test (treated)	20	20	19	20	21	20	0.70
Citrus limetta control	8	7	7	8	9	8	0.83
Fluconazole	12	12	12	12	12	12	0

Candida albicans is an opportunistic fungal pathogen and fluconazole is the preferred drug to treat Candida infections [19]. Drug resistance or decreased activity of antifungal agents after prolonged used is a common problem [20]. *Citrus limetta* flavonoids are known to have antifungal activity; enzymatic transformation may increase such activity and provide a new solution to cure candidiasis [6, 20]. The treated sample showed a 56.52% increase in the zone of inhibition against *Candida albicans* as compared to the control sample. Moreover, the zone of inhibition for the treated sample was significantly larger than that of fluconazole. This suggests that enzymatic modification increases the antifungal activity of these flavonoids significantly. Enzymatically modified flavonoids can serve as a natural substitute to solve the problem of fluconazole resistant Candida infections [20].

CONCLUSION

Enzymatic modification of flavonoids alters their bioactive principle and therefore may enhance their stability and solubility in various systems. Thus making them therapeutically more useful. Enzymatic modification of flavonoids from *Citrus limetta* results in significantly higher antifungal activity. Further study would involve performing LC-MS for treated and control samples in order to separate and determine the difference in the specific compounds present in the two for more reliable results.

CONFLICT OF INTERESTS

Declared None

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