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ANTI- BACTERIAL, ANTI- FUNGAL AND ANALGESIC ACTIVITY OF PAPAIN CONJUGATED QUERCETIN

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ABSTRACT
The flavonoids comprise a large class of low-molecular-weight plant metabolites ubiquitously distributed in food plants. These dietary antioxidants exert significant antitumor, antiallergic, and anti-inflammatory effects. Quercetin is a naturally occurring chemical found in fruits and vegetables. Flavonoids such as quercetins, are antioxidants, antibacterial and anti inflammatory agents. The molecular mechanisms of their biological effects remain to be clearly understood. Flavonoids have more therapeutic activity and less solubility. So, its activity can be enhanced through enzymatic conjugation or sulfation by Arylsulfotransferases or by other hydrophilic enzymes. In the present work, we have taken Papain conjugated Quercetin enzyme to increase its solubility and bioavailability. Enzyme conjugation was analysed by HPLC method. HPLC at 255nm using an isocratic mobile phase (60:40, acetonitrile: 0.1% acetic acid). HPLC was evaluated through high accuracy, precision, recovery And that enzymatic conjugation may enhance the antibacterial, analgesic activity but antifungal activity was found to be less.

KEYWORDS: Flavonoids, HPLC, enzymatic conjugation, bioavailability, papain.

INTRODUCTION
The most important dietary sources of flavonoids are citrus fruits (rutin and hesperidin), apple (quercetin), tea and soybean. Flavonoids are becoming very popular because they have much health promoting effects. Some of the activities attributed to flavonoids include anti-allergic[1] anti-cancer[2], antioxidant[3], anti-inflammatory[4] and anti-viral[5] The flavonoids quercetin is known for its ability to relieve hay fever, eczema, sinusitis and asthma. Soy flavonoids (isoflavones) can also reduce blood cholesterol and can help to prevent osteoporosis.[6] Flavonoids have been shown to have direct antibacterial activity, synergistic activity with antibiotics, and the ability to suppress bacterial virulence factors in numerous *in vitro* and a limited number of *in vivo* studies[7][8] Noteworthy among the *in vivo* studies[9-11] is the finding that oral quercetin protects guinea pigs against the Group 1 carcinogen *Helicobacter pylori*. Researchers from the European Prospective Investigation into Cancer and Nutrition have speculated this may be one reason why dietary flavonoid intake is associated with reduced gastric carcinoma risk in European women.[12]

QUERCETIN
Quercetin is a flavonoid widely distributed in nature. Quercetin has GRAS (Generally Recognized as Safe) status, and no side-effects have yet been noted in doses of a few grams a day in either humans or animals. Quercetin has anti-oxidant[13], anti-artherogenic[14], and anti-carcinogenic [15] properties. But quercetin having low oral bioavailability and it could be due to *in vitro* studies using a form of quercetin called 'quercetin aglycone' whereas this particular form is never found in the blood, even after ingested, as it gets changed in the liver.

After oral ingestion of quercetin, it is taken up from the gut into the liver. An enzyme conjugated quercetin influences its absorption rates. At least intestinally, quercetin glycosides (food source) were found to have a 52+/-15% uptake, quercetin rutinoside (tea) has a 17+/-15% uptake, and supplemental quercetin aglycone had a 24+/-9% uptake ([16]) So, in the present research work we conjugated quercetin with papain enzyme, conjugation was estimated by HPLC analysis and we studied the antibacterial, antifungal and analgesic activity of conjugated quercetin.

MATERIALS AND METHODS
Materials of enzyme conjugation (Papain with Quercetin)
Chemicals, animals and cultures
- Flavonoid quercetin was provided as a powder extract from sigma Aldrich, Inc. in a standardized, Good Manufacturing Practice formulation. The

enzyme papain also purchased from Sigma-Aldrich, Inc. The mixture contained quercetin (75%), papain conjugated quercetin [2%; verified by high-performance liquid chromatography (HPLC) assay in our laboratory]. All common chemicals used in this study were purchased from LOBA chemie, INDIA. The microorganisms and media components used in the bioassay were procured from national collection of industrial microorganisms(NCIM), Pune and Hi media Ltd, Mumbai, India. Albino Wister Rats (four in a group) were collected from ---- and Hot plate analgesic meter (HTC life sciences, USA).

Procedure for conjugation

Synthesis of the papain conjugated quercetin was achieved by modified method of *vijay kumar et al.* Quercetin (0.25gm) was dissolved in 1 ml of Dimethyl Sulfoxide (DMSO) with constant stirring, to which a phase transfer catalyst (0.1 M of benzyl triethyl ammonium chloride) dissolved in 10 ml of sodium acetate buffer (0.01 M, pH 7.5) was added. To the above reaction mixture, glucose (0.048 gm) and papain (0.024 gm) were added and incubated in shaking incubator at 40 ± 2^0 C, 150 rpm for 35 hrs. The product formation was intermittently monitored by HPLC. After completion, the reaction was quenched by keeping in a boiling water bath for 10 min. The product formed in the reaction mixture was concentrated by vaccum evaporation to get the crude product. The concentrated product was tested by HPLC.

Preparation of standard Stock solution

Quercetin**:** Weigh 2mg of Quercetin in a 10 mL volumetric flask. Add 8mL of diluent, sonicate to dissolve and dilute it with diluents at required volume.

Preparation of standard solution

Further, dilute each 5mL of the standard stock solution to 10 mL with the diluent.

HPLC

The concentrations of quercetin and Papain conjugated quercetin were analyzed by HPLC (Gilson LC system), using analytical Column: Luna C18, 250mm x 4.6mm, 5µm particle size, Injection Volume: 10µL, Column Temperature: Ambient, The system was run isocratically using mobile phase Acetonitrile and Water (60:40) (pH adjusted to 3.5with acetic acid) at a flow rate of 1.0mL/min and the sample detection was done using UV/VIS detector at 255 nm. The mobile phase was filtered through 0.45µ membrane filter and the solvent was degassed ultrasonically before use. Record the chromatograms and measure the peak responses for Quercetin. The System suitability parameters should be met. From the peak responses, calculate the content of Quercetin in the sample.Retention time of Quercetin is about 3.2 min.

Evaluation of system suitability

1. Relative Standard Deviation of five replicate injections of Standard preparation for Quercetin peak should not be more than 2.0%.
2. Tailing factor for Quercetin peak should not be more than 2.0%.
3. Theoretical Plate count for Quercetin peak should not be less than 2000.

Antimicrobial Assay

The antimicrobial activity of quercetin and papain conjugated quercetin was tested against *Staphylococcus Aureus (ATTC-2901)* and *Aspergillus Niger(ATTC-1196.)* Muller Hinton agar and Potato Dextrose Agar (PDA) were used to cultivate the bacteria and fungi. Each culture was transferred from agar slants into 10mL of broth and incubate overnight at 37^{0c} for bacteria and two days at 28^{0c} for fungi. 12hrs old bacterial and 48 hrs old fungal cultures were used for determining minimum inhibitory concentration (MIC).

Determination of MIC

The agar well diffusion method was used to assay the quercetin and papain conjugated quercetin for antimicrobial activity. The above prepared test cultures were seeded into sterile nutrient agar medium by uniformly mixing 1 ml of inoculums with 20 ml melted nutrient agar medium, cooled to $48\text{-}50^0$ C. and then allow it to solidify. Six wells (7 mm each) were bored in each plate using an aseptic well borer. The test compounds (quercetin and papain conjugated quercetin) and control samples (gentamicin against bacteria and fluconazole against fungi) were dissolved in 90% DMSO and serially diluted to get concentration of 2-100 ug/ml. an equivalent amount of DMSO (0.1mL) was added to each plate as a negative control. Each experiment was performed in duplicates and repeated thrice. The MIC was reported as the lowest concentration of test sample capable of inhibiting the growth of each bacteria and fungi used in the study.

Analgesic Activity

Animals used

Albino Wister rats of either sex weighing between (150-200 gms) were divided into groups of four animals and each group consists of four animals.

- Group 1: Positive Control
- Group 2: Negative Control
- Group 3: Standard Ibuprofen
- Group 4: Test (Enzyme-conjugated Quercetin)

The animals were maintained under laboratory condition and kept in standard cases at room temperature of 30 ± 2^0 C and 60 to 65% relative humidity and provided with standard diet and water.

The enzyme conjugated Quercetin was given to group 4 and the analgesic activity studies are conducted using Ibuprofen as standard. The method used is Hot plate method.

Hot plate method

The Hot plate method described by Woofle 7 MC Donald(1944) with slight modification (Santos et al., 1998) was followed to measure the central analgesic activity of papain conjugated Quercetin. Albino Wister Rats (four in a group) were placed on a hot surface of a plate instrument (Hot plate analgesic meter HTC life sciences, USA) maintained at 50 ± 5^0C. Time taken by the animals for shaking, licking (or) jumping due to pain was recorded as the indicator for response latency. Prior to the experiment, all the animals were made acquainted with the instrument and animals with response latency more than 30 sec were not used for the experiment. An automatic cut-off time of 45 sec was set in order to prevent any tissue damage of the Albino Wistar rats paw. The enzyme conjugated Quercetin, Free Quercetin, Standard Ibuprofen were administered orally. The response latency was measured at different time intervals after the administration of each sample.

Statistical analysis

All experiments were conducted in triplicates and the results were presented as the mean of
Three independent experiments +_ standard error.

RESULTS AND DISCUSSION

In the present study quercetin and conjugated quercetin were synthesized and charcterised by spectral analysis HPLC. Thus from the retention time and area under peak, it was confirmed the enzymatic conjugation was confirmed and reports were also given in the chtomatogram 1, 2 and 3. The enzyme conjugated quercetin can acts as potent prodrug as glucosyl bond gets hydrolysed at the target sites to liberate pharmacological active molecule quercetin. The present methodology develops involved a one step glucosyl; conjugation to quercetin by papain, which showed good yield (25%) and enantioselectivity. Despite quercetin demonstrated therap[eutic efficacy and safety, the poor bioavailability of its into systemic circulation continues to be highlighted as a major concern in wider pharmacological therapeutic appilications. From the literature, it is well known that the glycosylation of hydrophobic compounds allow the conversion of water insoluble compound into soluble compound, which could improve the bioavailability and pharmacological properties of the compound.

Quercetin is a bioactive compound , has been shown to have several biological active properties, such as anti oxidant, anti infectious, antiviral and anticancer .*In vitro* antimicrobial activity of quercetin and enzyme conjugated quercetin was compared with standard antibacterial and antifungal drugs such as gentamyci sulfate and flucanazole. Both the molecules quercetin and enzyme conjugated quercetin showed antibacterial activities with a range from. 0.6 -0.9mm respectively against the bacterial test culture but failed against fungi test culture.enzyme conjugated quercetin shon the best result [Fig:1], having lower MIC values compared to quercvetin[Fig:2] and control[Fig:3]anf results were shown in the [Table:1]Therefore the improved antibacterial activity of enzyme conjugated quercetin may because of its increased solubility, enhanced cellular uptake, reduced metabolism and better binding to cell components. And the antifungal activity was found to be nil [Fig:4].

Analgesic activity

Analgesic activity of quercetin against enzyme quercetin was compared with standard ibuprufen. Papain conjugated quercetin showed more response [Table:2, Graph:2] due to its improved bioavailability.

TABLES AND GRAPHS

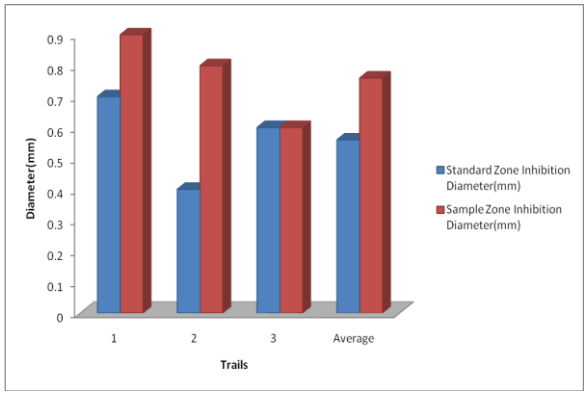
Table 1: Anti bacterial activity- quercetin vs enzyme conjugated quercetin

Trails	Standard (quercetin)zone inhibition diameter(mm)	Sample zone inhibition diameter(mm)
1.	0.7	0.9
2.	0.4	0.8
3.	0.6	0.6
Average	0.56	0.76

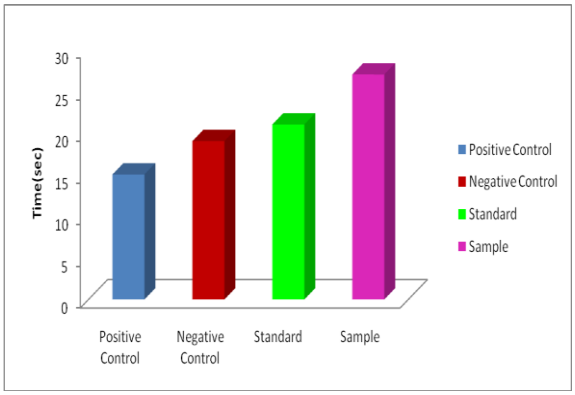
Antimicrobial Activity

Table 2: Analgesic activity - the time of response for enzyme conjugated Quercetin

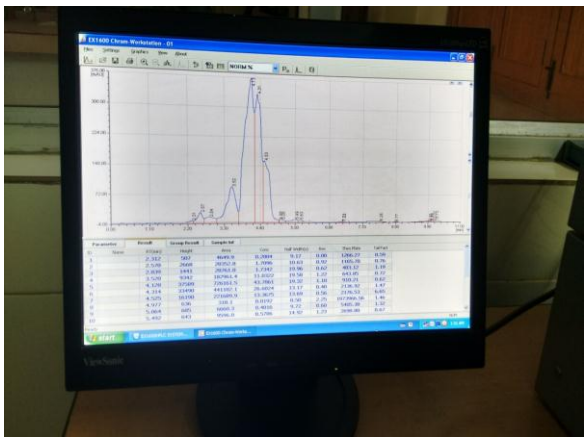
Group	Treatment	Response	Time(min)
Positive control	Normal saline	Paw licking, jumping	15
Negative control	Quercetin	Paw licking	19
Standard	Ibuprofen	Paw licking	21
sample	Enzyme conjugated Quercetin	Paw licking, jumping	27



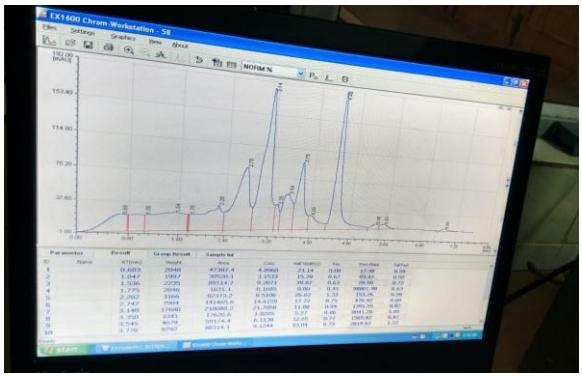
Graph 1: Antibacterial Activity of Quercetin Vs Conjugated *Quercetin*



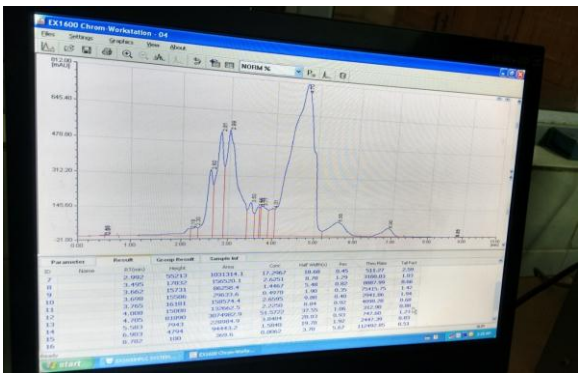
Graph 2: Analgesic Activity of Quercetin Vs Conjugated Querceti



Chromatogram 1: free quercetin with DMSO



Chromatogram 2: free quercetin with acetonitrile



chromatogram 3: Conjugated quercetin

Fig 1: Antibacterial activity of papain conjugated Quercetin.

Fig 2: Antibacterial activity of Free Quercetin.

Fig 3: Antibacterial activity of control

Fig 4: Anti- Fungal activity of Quercetin and Papain conjugated Quercetin

CONCLUSION

Quercetin was improved for its solubility and bioavailability by glycosilation to papain conjugated by a selective enzymatic route with good yields and enantioselectivity in a simple ecofriendly reaction condition. Increased solubility of papain conjugated quercetin results in its synergistic effect of enhanced anti oxidant , antimicrobial and analgesic activity, which can improve the medical value of quercetin. Thus the enzymatic conjugation of flavonoids may give a hope for future poential drug in the treatment of various microbial and other diseases.

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