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Phytochemical Investigation and Pharmocological Studies of the Flowers of *Pithecellobium dulce*

P.G.R. Chandran[1] and S. Balaji[2]

[1]Lecturer in Chemistry, School of Chemical and Biotechnology, SASTRA University, Thanjavur, Tamilnadu-613402, India

[2]Lecturer in Bioinformatics, Department of Biotechnology, Manipal Institute of Technology, Manipal University, Manipal-576104, India

[2]Corresponding Author: Tel: mobile-9895592347
E-mail: blast_balaji@rediffmail.com

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ABSTRACT

To evaluate the effects from the fresh flowers of *Pithecellobium dulce* (Roxb.) Benth, belonging to the family of Leguminosae subfamily Mimosoideae, a glycoside quercitin has been isolated. The ethyl acetate soluble of *P. dulce* containing the above glycoside was studied both *in silico* and *in vitro* for the anti-inflammatory and anti-bacterial properties. The concatenation of the *in silico* and *in vivo* has been done. Results indicated the activity of this flavonol glycoside in the protection of HRBC lysis and against the gram positive micro organisms, thus confirming its anti-inflammatory and anti-bacterial properties.

INTRODUCTION

Pithecellobium dulce (Roxb.) Benth, belonging to the family of Leguminosae, subfamily Mimosoideae, is a small evergreen thorny tree[1]. The tree is reported to be active against veneral diseases[2]. The decoction is given as enema[3]. The seeds contain saponin[4]. As there is no work on the flowers of *P. dulce*, the flowers of same have now been examined for their poly-phenolic constituents. The crude extract of the flowers has also been investigated *in silico* and *in vitro* for their anti-inflammatory and anti-bacterial properties and our results are presented in this communication.

METHODOLOGY

From the fresh flowers (300g) of *P. dulce* collected from Thanjavur district of Tamilnadu, India, during February, were dried in shade and extracted with 80% ethanol in cold. The combined alcoholic extract was concentrated in vacuum and the aqueous concentrate was successfully fractionated with benzene, peroxide free Et_2O and EtOAc. The benzene fraction did not yield any crystalline solid. The residue from the EtOAc fraction was taken up in minimum quantity of Me2CO and left in an ice chest for about a week when yellow solid separated. On re-crystallization from aqueous methanol, it came out as pale

yellow needles, m.p 313-315C (yield 0.01 %).

λ_{nm}^{max} : 255,269sh, 370 ;(MeOH+NaOMe):262sh,321,420(dec.);(MeOH+$AlCl_3$): 267,303,458 ;(MeOH+$AlCl_3$+HCl):267,303,351,428;(MeOH+NaOAc): 275,328,390 ;(MeOH+NaOAc+H_3BO_3):262,303sh, 386;

red color with Mg-HCl[5], Olive green color with alc.Fe^{3+}, golden yellow color with NH_3 and NaOH, yellow solution with pale green fluorescence with conc.H_2SO_4, yellow under UV/NH_3, responded to Horhammer-Hansel **[19]**, Wilson's boric acid test **[20]** and Gibbs test**[21]** test indicative of a flavonol 3-O glycoside and the identity was confirmed by co- and mixed PC with an authentic sample of quercetin.

The ^{13}C- NMR data of the isolated compound is given as follows:

^{13}C-NMR (125MHz,DMSO-D_6,TMS) d ppm:157.3(C-2),134.2(C-3),178.1(C-4).162.1(C-5),99.5(C-6),165.0(C-7),94.3(C-8),157.0(C-9),104.8(C-10),121.8(C-1^1),116.0(C-2^1),145.6(C-3^1),148.9(C-4^1),116.0(C-5^1) and 122.0(C-6^1)

The UV and 13C- NMR spectral data were in agreement with the flavonol quercetin.

A solution of the glycoside was hydrolyzed (7%H_2SO_4, 100° C, 2 hr.) and the aglycone was characterized as quercetin (m.m.p, uv data, Rf, acetate and methyl ether) and the sugar was identified as rhamnose. A quantitative hydrolysis of the same by the Folin-wu's micro method **[22]** revealed to be a monoside. The glycoside was thus characterized as quercetin 3-O rhamnoside (quercitrin) and the identity was confirmed by direct comparison with an authentic sample of quercitin. Based on the UV and 13C- NMR spectral data, it is crystal clear that the isolate is flavonol quercetin. The structure of the quercitrin was drawn by using ACD-3D ChemSketch.**[5]** Then we performed the conversion of the drawn chemical structure into SMILES **[7]** notation, so as to predict biological activity and to find similar chemical compounds. The SMILES notation used for screening similar compounds and biological activity is given below.

C5(O)C(O)C(O)C2[O+]([H-]C1C(O)C(O)CCC1C3C(O2)C(O)C4C(O)CC(O)CC4O3)C5

To find similar chemical compounds we have screened the compounds from NCI. [8] For predicting biological activity we used PASS. [6]

RESULTS AND DISCUSSION

Quercetin 3-O-rhamnoside (quercitrin) see figure 1, has been isolated from the fresh flowers of *P. dulce*. The UV spectrum of the glycoside exhibited two major absorption peaks at 350 nm (band I) and 256 nm (band II). The band I absorption of the glycoside is reminiscent of a flavonol skeleton. A comparison of band I absorption of the glycoside and that of the aglycone revealed that there may be 3-glycosilation in the flavonol. A bathochromic shift of 43 nm (band I) in NaOMe confirmed the presence of a free –OH at C-4^1 .The $AlCl_3$ spectra (with and without HCl) showed four absorption peaks to reveal the presence of a free 5-OH group. It was confirmed by the bathochromic shift of 50 nm on the addition of $AlCl_3$-HCl in the glycoside. The presence of a free –OH group at C-7 was evident from the +16 nm (band II) shift on the addition of NaOAc. The band I absorption in $AlCl_3$ spectrum is 30 nm more than that noticed on addition of $AlCl_3$-HCl.This is indicative of the existence of an O- dihydroxyl group in the B-ring. In the ^{1}H-NMR spectrum (400MHz,DMSO-D_6,TMS) the signal at d 6.47 and 6.42 ppm corresponds to the A-ring protons at C-8 and C-6.The 5_OH protons resonates at d 12.56 ppm.The proton C-5^1 appears at d 6.86 ppm as a doublet. The signal d 10.88ppm can be traced to the-OH at C-7 and C-2^1.The C-6^1 protons show up at d 7.30 ppm.The methyl protons of rhamnoside moiety resonate at d1.18 nppm and the H-1 of rhamnoside resonates at d5.31 ppm.The remaining sugar protons appear in the range of d3.37-4.0 ppm Supporting evidence for the structure of the glycoside was provided by the analysis of 13C-NMR (100MHz, DMSO-D6, TMS) data. Due to glycosilation at 3-position, the C-2 and C-4 carbons absorb at d 157.3 and 178.1 ppm respectively.

Figure 1: Quercitrin structure.

The stick model shown in this picture (color by CPK) is based on the actual chemical structure provided

by the analysis of 13C-NMR (100MHz, DMSO-D6, and TMS) data.

Thus on the basis of the above mentioned Physical and Chemical evidences the glycoside obtained from *P. dulce* has been characterized as quercetrin. Biological activity prediction is pivotal in any structure prediction. Based on the UV and 13C- NMR spectral data, it is crystal clear that the isolate is flavonol quercitrin. We were much interested to predict its biological activity. So as to predict structure activity relationship, for that the structure of the quercitrin was drawn by using ACD-3D ChemSketch v 5.12 [5]. We have performed *in silico* studies like chemical structure similarity search and the Prediction of Activity Spectra for Substances: PASS [6] prediction was done by converting the quercetin structure into the Simplified Molecular Input Line Entry System (SMILES notation) [7] proposed by Dave Weininger (Weininger, 1988). Finally we have found some similar structures in enhanced NCI database browser release 2. [8] The obtained quercitrin structure was submitted for Pass prediction and we obtained the 41 substructure chemical descriptors. We have taken Pa >0.7 and found that the highest hit had predicted membrane integrity agonist and anti-inflammatory activities. Because if the Pa >0.7, the substance is very likely to exhibit the activity in experiment. The list of predicted properties by PASS Pa >0.7 is given in the Table 1. The reliable effects and mechanisms are listed in Table 2, and 3.

Table 1: PREDICTED ACTIVITY

Pa	Pi	Activity
0,969	0,004	Membrane integrity agonist
0,891	0,004	Membrane permeability inhibitor
0,885	0,006	Vascular (periferal) disease treatment
0,849	0,002	Capillary fragility treatment
0,751	0,008	Topoisomerase II inhibitor
0,731	0,007	Emetic
0,728	0,006	Sweetener
0,725	0,005	Osmotic diuretic

Table: 2. PREDICTED RELIABLE EFFECTS.

Pa	Pi	Activity:
0.969	0.004	Membrane integrity agonist
0.969	0.004	Antiinflammatory
0.969	0.004	Antibacterial
0.969	0.004	Psychotropic
0.969	0.004	Antiepileptic
0.969	0.004	Immunostimulant
0.969	0.004	Antiviral (HIV)
0.969	0.004	Antiviral (herpes)

0.969	0.004	Antineoplastic
0.969	0.004	Antiprotozoal
0.969	0.004	Dermatologic
0.969	0.004	Antieczematic
0.969	0.004	Antiseborrheic
0.969	0.004	Antiischemic
0.969	0.004	Antiischemic renal

Table: 3. PREDICTED MECHANISMS

Pa	Pi	Activity
0.969	0.004	Membrane integrity agonist
0.969	0.004	Antiinflammatory
0.969	0.004	Antineoplastic
0.969	0.004	Antifungal
0.969	0.004	Antiviral
0.969	0.004	Antiseborrheic
0.969	0.004	Antiviral (HIV)

So from the *in silico* predicted information, we have decided to test the activity *in vitro* especially for the membrane permeability inhibitor (Table-1), membrane integrity agonist (Table 1, 2, 3), anti-bacterial effects(Table-2) and anti-inflammatory mechanisms (Table-2 & 3).

ANTI-INFLAMMATORY STUDIES

Lysosomal enzymes play an important role in the development of acute and chronic inflammation.[9] Increased enzyme activity has been reported in certain types of experimental inflammation.[10] The inhibitory effects of non-steroidal anti–inflammatory drugs on lysosomal enzymes have been proposed as an explanation for one of their many mechanisms of actions *in vitro.*[11] Acidic anti-inflammatory compounds such as phenyl butazone, Mefenamic acid and indomethacin have been shown to exert their beneficial effect by inhibiting the activities of either released lysosomal enzyme or by stabilizing the lysosomal membrane[12-14]. It has been reported that the structure of RBC is similar to that of lysosomal membrane components.[15] Since lysosomal membranes resemble human RBC (HRBC) membranes, the lysosomal membrane effects have been studied using HRBC. When the RBC is subjected to hypotonic stress, the release of haemoglobin from RBC is prevented by anti-inflammatory drugs because of the membrane stabilization. Hence the HRBC membrane stabilization by drugs against hypotonicity induced haemolysis serves as a very useful *in vitro* method for assessing the anti-inflammatory activity of compounds. In this present investigation, an *in vitro* study of the EtOAc isolates of *P.dulce* by finding

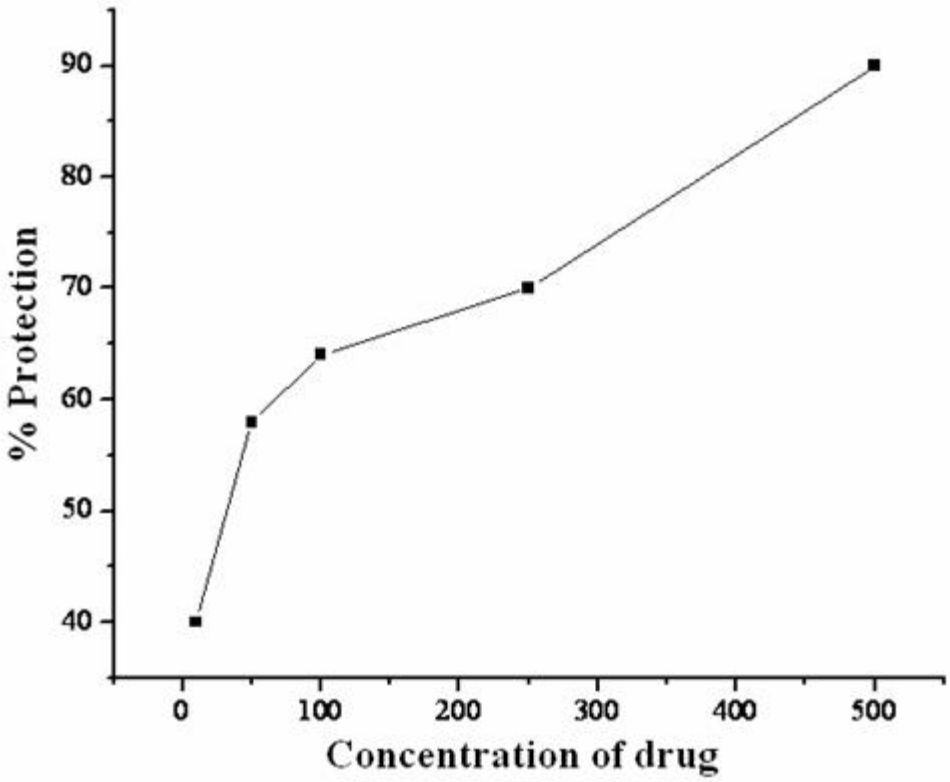
the stabilization of the HRBC membrane against hypotonicity induced haemolysis has been made,[16] the results are indicated in the Table 4 and the Graph-I. From the results the *in silico* predicted membrane permeability inhibitor (Table-1), membrane integrity agonist (Table 1, 2, 3), and anti-inflammatory mechanisms (Table-2 & 3) have been understood *in vitro.*

Table: 4: Stabilization Effect of isolates of *P.dulce* on the HRBC membrane stabilization against hypotonicity induced haemolysis.

Sl. No	**Concentration of the drug in** m**g**	**Percentage protection**
1	10	40
2	50	58
3	100	64
4	250	70
5	500	90

Based on the *in vitro* Stabilization Effect of isolates of *P.dulce* on the HRBC membrane stabilization against hypotonicity induced haemolysis, the tabular values are plotted as a graphical representation. (Graph-I)

Graph-I: The *in vitro* Stabilization Effect of isolates of *P.dulce* on the HRBC membrane stabilization against hypotonicity induced haemolysis. The concentration of the drug (in m**g)** used in the protection of HRBC membrane is plotted in the graph.



The crude extract was observed to be effective in stabilizing the HRBC membrane against hypotonicity induced haemolysis and hence would be effective as non steroidal anti-inflammatory compounds in the control of inflammation. With in the experimental range of dosages of (10 to 250 mg /ml) the flavonoid drug exhibited 70% protection at 250 mg dose and at subsequent doses, the protection increases and reached a maximum with a sharp increase at 500 mg. At higher concentrations the activity climbs up showing the anti-inflammatory activity of this flavonoid drug under in vitro experimental conditions dependent upon the concentration of the drug. The membrane is stabilized by theflavonoidal drug at a concentration of 500 mg.

ANTI-MICROBIAL STUDIES

In this investigation , the anti- bacterial activity of the residue of the EtOAc fraction containing the flavonoid glycoside isolated from the flowers of *P.dulce* have been studied *in vitro* by Petri-dish method using *Staphylococcus aureus* a gram positive, Escherichia coli and *Salmonella typhii* two gram negative as test organisms. Anti-bacterials produce their effect by interfering with one or more vital metabolic pathways in the organism. The object of the treatment with an anti-microbial drug which is higher than the minimal effective concentration and which is maintained at that level until the organisms have been eliminated [17].The extracts of various medicinal plants containing flavonoids have been reported to possess anti- bacterial activity [18].A standard volume (2.5mL) of Mueller-Hinton agar medium that would support the growth of the test organisms was added to sterile Petri–dishes. Solutions of the test

compound (EtOAc residue) at six different concentrations viz., 25, 50,100,200,300 and 400 mg/mL in sterile water were prepared. Standards containing streptomycin at concentration of 50,100 and 200 mg/mL and a control containing no drug were prepared. A standard inoculum of a suspension of turbidity equal to a McFarland standard 0.5 of the test organism was added to all Petri–dishes. After inoculation, the plates were incubated at 37°C and minimum inhibitory concentration (MIC) is found out after 48 hours of incubation. The number of colonies that grow on this subculture is then counted and compared to the number of CFU/mL (Colony Forming Units) in the original inoculum. In the anti-microbial studies only traces of the growth has been observed at a lower concentration of the drug. The growth of the organism is inhibited with higher concentration.

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