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HPTLC Analysis and In-Silico Studies of Three Bioactive Natural Colors from Cissalpenea Sappan Wood, Clitorea Ternatea Flowers, and Selenicereus Undatus Fruits

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ABSTRACT

The work focusses on, an ecofriendly and cost-effective extraction of three natural colorants obtained from Caesalpinia sappan, Clitoria ternatea and Selenicereus undatus which can be used as pharmaceutical excipients, food colorants, and cosmetics, etc. Apart from that the natural color itself has its own therapeutic activity therefore, the rational use of these colors along with the synthetic drugs will impart not only the coloring effect for the formulation but it can make an additive effect along with the therapeutic action of the drug.

HPTLC evaluation of these colour extracts indicated the presence of the reference standard quercetin in Clitoria flower and Sappan wood extracts as 87.68 mcg/mg and 72.64 mcg/mg respectively. The amount of reference standard gallic acid in dragon fruit extract is found to be 38.46 mcg/mg.

The colour extracts CSWC (C. sappan wood color extract), CTFC (C. ternatea flower colour extract) and SUFC (S. undatus fruit colour extract) showed an LC50 value of 7943.3, 6534.5 and 5623.4 mcg/ml respectively as compared with FCF color 15985,14720 (3981.07mcg/ml), Brilliant blue FCF (3771.7mcg/ml), Carmoisine (1989.57 mcg/ml) respectively in the brine shrimp lethality assay method (BSLA). The percentage yield obtained for CSWC, CTFC, and SUFC are 90.5, 94.25 and 95.35 respectively.

In-silico molecular docking studies revealed that quercetin present in CSWC, CTFC and gallic acid present in SUFC showed significant binding affinity against COX-2, DPP-4 and SOD respectively.

***Key words**: CSWC, CTFC, SUFC, HPTLC, BSLA*

1. INTRODUCTION

Colour is regarded as the fundamental characteristic that the senses perceive, and it has played a significant part in the acceptance of foods for ages by improving their real appearance and quality [1]. The plant pigments, have a great deal of promise to replace many artificial colorants.

The several kinds of natural colours that give foods their diverse colour tints are betalains, carotenoids, anthocyanins, and chlorophylls, [2,3]. The use of plant pigments in the food sector has several positive aspects. Natural dyes are not harmful, carcinogenic, polluting, or toxic.

Natural colouring agents are compounds that come from natural resources and are used to colour pharmaceuticals, foods, fabrics, and cosmetics. Unlike synthetic dyes, these agents are typically extracted from plants, minerals, or insects and are often preferred for their perceived safety and environmental benefits [3].

Usually, different types of synthetic colors are used in pharmaceutical, nutraceutical and herbal products as coloring agents. The manufacture of these synthetic colorants was estimated to release high levels of

non-degradable waste which are unsafe to human health, and environment. The World Health Organization specified and set allowable limits for the use of these synthetic colors due to their toxicity in different organ systems [4]. Natural color excipients are substances derived from natural sources used to add color to foods, textiles, cosmetics, and pharmaceutical products [5,6,7,8]

The plants selected for the extraction of colours are Caesalpinia sappan heart wood (sappan wood) [9,10], Clitoria ternatea flower (butterfly pea) [11,12,13,14,15] and Selenicereus undatus fruit (Dragon fruit) [16,17,18] which are decided to process by the methods like solvent extraction, filtration, concentration, spray drying, milling and sifting respectively.

The extraction of all the three colors by a solvent mixture comprises of water and ethanol in the ratio of 50:50, 60:40, 70:30 and 80:20 as a trial and whichever solvent mixture ratio gives maximum percentage yield will be selected for the final extraction. The extracted colors are quantified for the presence of therapeutic / analytical marker compounds by HPTLC analysis.

The present work focusses on the extraction and HPTLC quantitation of major phytocompounds present in the selected plant parts. Also, the work aims to develop toxicological data and In-silico profiles by molecular docking techniques for these natural colors.

2. MATERIALS AND METHODS

2.1. Methods of extraction and processing of the colour extract

Common extraction procedure for the plant parts selected for all the three plant parts (Caesalpinia sappan heart wood (Biancaea sappan), Clitoria ternatea flower (butterfly pea), and Selenicereus undatus fruit (Dragon fruit) selected is as follows

1. Harvesting and drying: Collect Clitoria flower petals and dry them immediately to preserve the color and prevent spoilage. Drying: Air drying
2. Extraction: Steep the dried petals in a solvent like water, ethanol, to release the pigment. Heat can be applied to enhance extraction
3. Filtration: Separate the liquid extract from the solids using a filter or cheesecloth.
4. Concentration: Evaporate the solvent using heat, vacuum, or freeze-drying to concentrate the pigment.
5. Spray drying: Convert the concentrated liquid into a dry powder using spray drying or freeze-drying techniques
6. Milling: Grind the resulting powder into a finer texture using a mill or grinder
7. Sifting: Sift the powder to remove any lumps or large particles.

2.1. Clitoria ternatea (Shankpushpi) F: Fabaceae**.** [11,12,13,14,15]

2.1.1. Collection and authentication

Clitorea ternata (F: Fabaceae) flower was collected in the month of May 2024 from a local nursery at Coimbatore and authenticated at the Botanical survey of India, The Tamil Nadu Agricultural university (TNAU), Coimbatore, (BSI/SRC/5/2024-25/Tech/991**)**. The sample specimens of the plant (specimen no. PPG.COG/112/2024) were deposited at the herbarium of the Dept. of Pharmacognosy, PPG College of Pharmacy, Coimbatore, 641035.

Water and ethanol solvent system at different ratios are used for extraction of Clitoria ternatea flower petals. The ratios and percentage obtained are tabulated. The percentage yield obtained for CTFC (Clitoria ternatea Flower colour) is 94.25 %. Table .1

Table 1. Analysis of the extraction patterns of C. ternatea flowers utilizing varying ratios of water and ethanol.

S. No	Water	Ethanol	% yield
1	50	50	74.5
2	60	40	79.8
3	70	30	85.45
4	80	20	94.25

The maximum percentage yield was obtained at the ratio of 80:20

2.1.2. HPTLC evaluation of Clitoria ternatea flower extract

2.1.3. HPTLC specifications

The sample solution for HPTLC was prepared by mixing 1g of the powdered dry petals with 10 ml of methanol and sonicated for 10 min. The mixture was then centrifugated to obtain a clear supernatant, which is used as test solution (10 mg/ml).

10 mg/ ml of Quercetin was dissolved in methanol and different amount of these were loaded into TLC plate. (2.0 -10 µl) which was fixed as the reference standard.

Pre dosage volume: 0.20 µl [application volume], Stationary phase: Merk, HPTLC Silica gel 60 $F_{254,}$ Mobile phase: Toluene: Ethyl acetate: Formic acid (5:5:1), Detection: UV wavelength 254 nm. The HPTLC quantitation results of the colour along with the marker compound are tabulated in Table .2 , Fig.1,2,3,4,5,6.

Table.2. HPTLC quantitative analysis of C. ternatea with the marker compound quercetin

Name of the plant	Parts of the plant used	Chemical marker	Solvent system	Rf value	Quantity present
Clitoria ternata	Flowers	Quercetin	Toluene: Ethyl acetate: Formic acid [5:5:1]	0.474	87.68mcg/mg

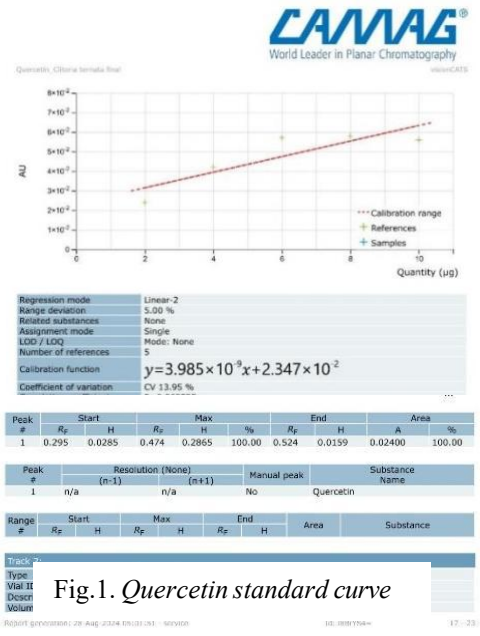


Fig.1. *Quercetin standard curve*

Fig.2.HPTLC: *Clitoria ternatea* Flower

Fig.4. HPTLC: *Clitoria ternatea* Flower

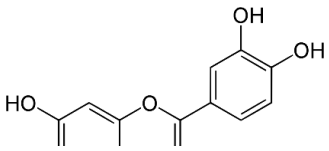


Fig.5.Quercetin

OH O

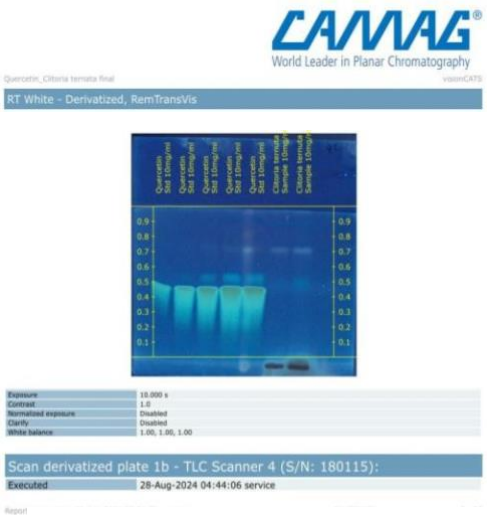


Fig.6.HPTLC: *Clitoria ternatea* Flower extract along with marker Quercetin

2.2. **Selenicereus undatus** fruit (Dragon fruit) F: Cactaceae [16,17,18]

2.2.1. Collection and authentication

Dragon fruit was collected in the month of May 2024 from a local nursery at Coimbatore and authenticated at the Botanical survey of India, The Tamil Nadu, Agricultural university (TNAU), Coimbatore, Tamil Nadu (BSI/SRC/5/2024-25/Tech/992).

The sample specimens of the plant (specimen no. PPG.COG/113/2024) were deposited at the herbarium of the Dept. of Pharmacognosy College of Pharmacy, Coimbatore, 641035.

2.2.2. Methods of extraction and processing of the colour extract: Common extraction process was carried out as discussed in section 2.1. Water and ethanol solvent system at different ratios are used for extraction of Selenicereus undatus Fruit. The ratios and percentage of Selenicereus undatus Fruit Color extract (SUFC) obtained are tabulated in Table 3. The data showed maximum percentage yield of 95.35 at the ratio of 80:20

Table.3. Analysis of the extraction patterns of Selenicereus undatus fruits utilizing varying ratios of water and ethanol.

S. No	Water	Ethanol	% yield
1	50	50	79.5
2	60	40	82.55
3	70	30	87.45
4	80	20	95.35

2.2.3. HPTLC SPECIFICATIONS

Source: Dried fruit extract of Selenicereus undatus Family: Cactaceae

Sample preparation: 1g of the dried and powdered fruit pulp was mixed with 10 ml of methanol and sonicated for 10 min. The mixture was then centrifugated to obtain a clear supernatant, which is used as test solution (10 mg/ml). 10 mg/ ml of Gallic acid (Reference standard) was dissolved in methanol and different amount of these were loaded into TLC plate. (0.5-2.5 µl)

Pre dosage volume: 0.20 µl [application volume], Stationary phase: Merk, HPTLC Silica gel 60 F_{254}, and Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.8:0.2) was used as the mobile phase, Detection: 10% Ethanolic sulfuric acid followed by Folin Cio calteu reagent, UV wavelength 254 nm. The HPTLC quantitation results of the colour along with the marker compound are tabulated in Table .4 , Fig.7, 8,9,10, 11,12.

Table 4. HPTLC quantitative analysis of S. undatus with the marker compound quercetin

Name of the plant	Parts of the plant used	Chemical marker	Solvent system	Rf value	Quantity present

Selenicereus undatus	Fruit	Gallic acid	Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.8:0.2)	0.45	38.46mcg/mg

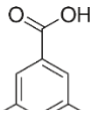


Fig.7. Gallic acid

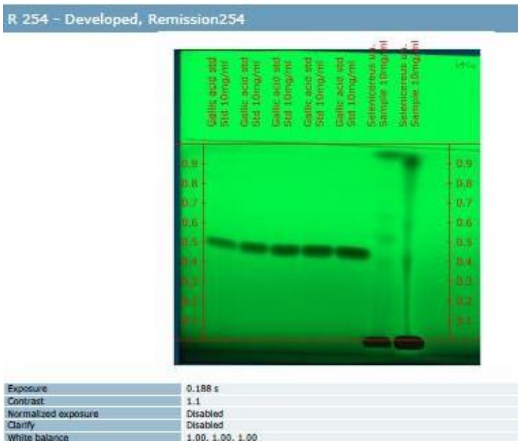


Fig.8. *S. undatus extract with Gallic acid marker*

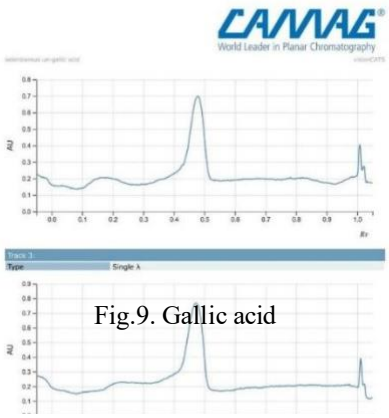


Fig.9. Gallic acid

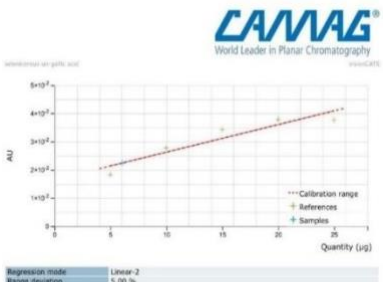
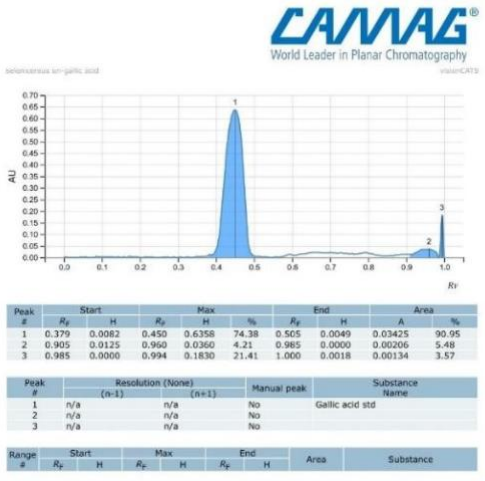


Fig.10. Standard curve: Gallic acid



Peak #	Start R_F	Start H	Max R_F	Max H	Max %	End R_F	End H	Area A	Area %
1	0.379	0.0082	0.450	0.6358	74.38	0.505	0.0049	0.03425	90.95
2	0.905	0.0125	0.960	0.0360	4.21	0.985	0.0000	0.00206	5.48
3	0.985	0.0000	0.994	0.1830	21.41	1.000	0.0018	0.00134	3.57

Peak #	Resolution (None) (n-1)	Resolution (None) (n+1)	Manual peak	Substance Name
1	n/a	n/a	No	Gallic acid std
2	n/a	n/a	No	
3	n/a	n/a	No	

Range #	Start R_F	Start H	Max R_F	Max H	End R_F	End H	Area	Substance

Fig.11.Gallic acid

Peak #	Start R_F	Start H	Max R_F	Max H	Max %	End R_F	End H	Area A	Area %
1	0.000	0.0000	0.008	0.1006	9.11	0.016	0.0000	0.00091	2.10
2	0.369	0.0203	0.429	0.4247	38.46	0.479	0.0139	0.02248	51.82
3	0.497	0.0182	0.529	0.0401	3.63	0.545	0.0279	0.00152	3.50
4	0.735	0.0532	0.774	0.0721	6.53	0.810	0.0573	0.00471	10.86
5	0.879	0.0709	0.940	0.1825	16.53	0.973	0.0551	0.01096	25.26
6	0.973	0.0551	0.981	0.2841	25.73	1.000	0.0001	0.00280	6.45

Fig.12. *Selenicereus undatus* fruit colour extract

2.3. **Caesalpinia sappan** heart wood **(**F: Caesalpiniaceae) [9,10,19,20,21]

2.3.1. Collection and authentication

Caesalpinia sappan wood was collected in the month of May 2024 from the College medicinal garden and authenticated at the Botanical survey of India, The Tamil Nadu, Agricultural university (TNAU), Coimbatore, Tamil Nadu (BSI/SRC/5/2024-25/Tech/993**).** The sample specimens (specimen.no.

PPG.COG/114/2024) were deposited at the herbarium of the Dept. of Pharmacognosy, PPG College of Pharmacy, Coimbatore, 641035.

2.3.2. Methods of extraction and processing of the colour extract: Common extraction process was carried out as discussed in section 2.1. Water and ethanol solvent system at different ratios are used for extraction of Caesalpinia sappan wood**.** The ratios and percentage obtained of Caesalpinia sappan wood Colour extract (CSWC) are tabulated in Table-5. The data showed maximum percentage yield of 90.5 for CSWC at the ratio of 80:20.

Table.5. Analysis of the extraction patterns of Caesalpinia sappan wood utilizing varying ratios of water and ethanol.

S. No	**Water**	**Ethanol**	**% yield**
1	50	50	75.8
2	60	40	77.45
3	70	30	86.5
4	80	20	90.5

2.3.3. HPTLC SPECIFICATIONS

Source: Dried Caesalpinia sappan wood extract of Family: Caesalpiniaceae

Sample preparation: 1g of the dried and powdered heart wood was mixed with 10 ml of methanol and sonicated for 10 min. The mixture was then centrifugated to obtain a clear supernatant, which is used as test solution (10 mg/ml).10 mg/ ml of quercetin (Reference standard) was dissolved in methanol and different amount of these were loaded into TLC plate. (0.5-2.5 µl), Pre dosage volume: 0.20 µl [application volume] Stationary phase: Merk, HPTLC Silica gel 60 F_{254}, Mobile phase: chloroform: methanol: water (64:50:10), Detection: UV wavelength 254 nm. The HPTLC quantitation results of the colour along with the marker compound are tabulated in Table .6, Fig.13, 14,15, and 16.

Table .6. HPTLC quantitative analysis of C. sappan with the marker compound quercetin

Name of the plant	Parts of the plant used	Chemical marker	Solvent system	Rf value	Quantity present
Cissalpenea sappan	Heart wood	Quercetin	chloroform: methanol: water [64:50:10]	0.945	72.64 mcg/mg

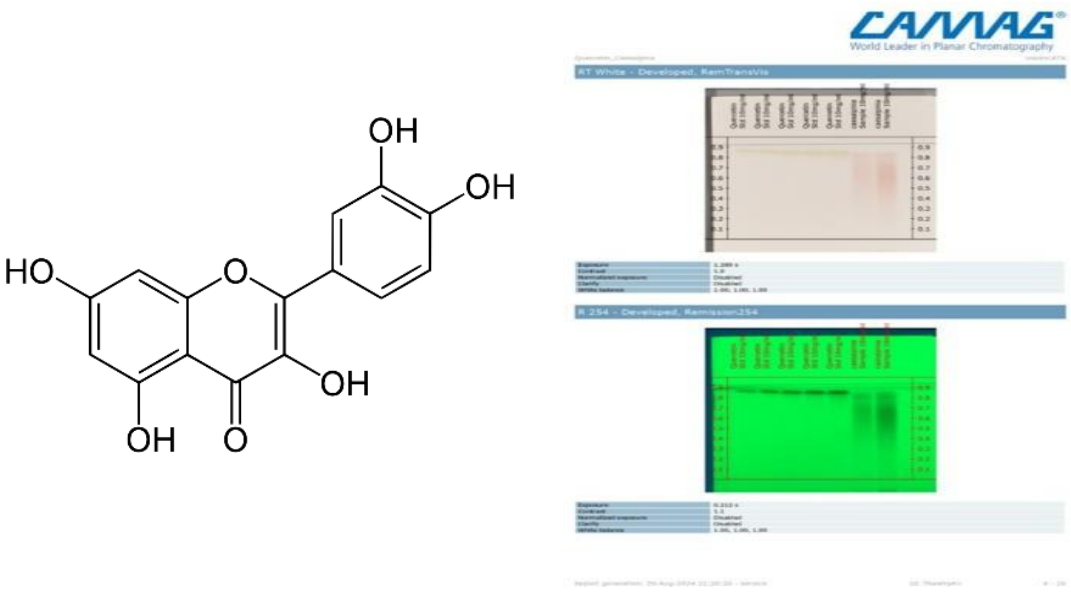


Fig.13.Quercetin and its HPTLC

Fig.14. Standard curve of quercetin

Fig.15, Fig .16. HPTLC of Quercetin and *C. sappan* red dye along with quercetin

Preliminary Cytotoxicity Studies [22,23,24,25]

This study [22,24] was performed using Brine Shrimp Lethality Assay (BSLA) method. The brine shrimps' eggs procured from the local aquarium and hatched with artificial sea water. Twenty numbers each of hatched brine shrimp (nauplii) were transferred to test tubes using a pipette. The survival rate of the brine shrimps was observed after 24h for various concentrations of CSWC, CTFC, SUFC and the synthetic counter parts colour Gold Camel FCF 15985, 14720, Brilliant blue FCF, and Carmoisine. The LC50 values for these colours were calculated using the dose response graph. The results are tabulated in Table 7.

Preliminary toxicity study using Brine shrimp assay method for Clitoria ternata blue dye, Caesalpinia sappan red dye & Selenicereus undatus red dye. Probit Analysis is used to determine the relative toxicity of chemicals to living organisms. This is done by testing the response of an organism under various concentrations of each of the chemicals in question and then comparing the concentrations at which one encounters a response.

Table.7. Percentage deaths of nauplii at 24 hr with different concentrations of colour extracts

Colour Extracts	0.01 mcg/ml	0.1 mcg/ml	1.0 mcg/ml	10.0 mcg/ml	100 mcg/ml	LC_{50} mcg/ml
CSWC	0	10	20	20	40	7943.3*
CTFC	0	10	20	20	35	5623.4
SUFC	0	15	25	30	55	6534.5*
Gold camel FCF color15985,14720	15	25	30	40	60	3981.07
Brilliant blue FCF	10	15	35	55	65	3771.17
Carmoisine	10	25	30	45	70	1989.57

n=20, CSWC: Caesalpinia sappan wood colour, CTFC: Clitorea ternata flower colour, SUFC: Selenicereus undatus fruit colour, student t test, p value <0.05 *, p value < 0.01: **

Dose Response graphs[22,23] for CSWC, CTFC, & SUFC

The percentage mortality was converted to probit units by the use of Finney's table then the log concentrations was calculated. A graph for the line of regression was plotted using probit versus log concentrations. The LC50 was calculated by finding the probit of 5 in the y-axis, and from the log concentration associated with it in the x-axis. The inverse of the log concentration was taken as the LC50.The results are tabulated in Fig.16,17,18.

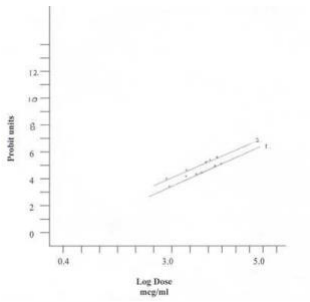


Fig.16. LC50 value *C. sappan dye* :7943.3mcg/ml
LC50 value: Gold camel FCF color 15985,14720: 3981.07 mcg /ml

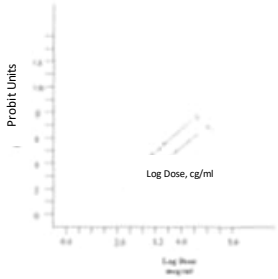


Fig.17. LC50 value *S. undatus dye :5623.4 mcg/ml*
LC50 value Carmoisine: 1989.57 mcg /ml

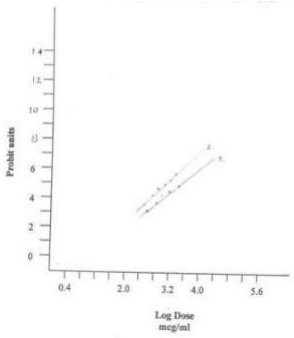


Fig.18. LC50 value *C. ternata dye :6534.5 mcg/ml*
LC50 value Brilliant blue FCF: 3771.17 mcg /ml

CSWC: Caesalpinia sappan wood colour, CTFC: Clitorea ternata flower colour, SUFC: Selenicereus undatus fruit colour

Table.8. Factors influencing the stability of the extracted natural colours (CTFC, CSWC, SUFC)
CTFC: C. ternatea, CSWC: C. sappan, SUFC: S. undatus

3. In-silico Molecular docking studies

The phytocompounds present in the plant extracts (quercetin and gallic acid) were docked by using Cyclooxygenase-2, Dipeptidyl peptidase- 4 and Superoxide dismutase (Protein Data Bank id 3MDL, 1J2E, 1IDS respectively.) The In-silico molecular study of all the selected compounds are found to obey "Lipinski rule of five". Autodock 4.2, Cygwin and Schrodinger software were used to predict the anti-inflammatory, anti-diabetic and antioxidant activities of the markers selected for the three colours. Molinspiration (online server) was used to evaluate the molecular properties of the selected markers (quercetin and gallic acid) selected. Table -9, Table-10, Fig. 19, 20, 21.

Table 9. PDB IDs of enzymes used in docking

Name of the colour/source	Influencing factors					
	Light	Temperature	pH		Oxidation	Solubility in water
CTFC C.ternatea	Stable at ordinary light	Stable at room temperature	Acidic	pink	Colour Fading	Highly soluble
			Alkaline	Purple		
			Normal	Blue		
CSWC C.sappan	Stable at ordinary light	Stable at room temperature	Acidic	Yellow	Red to brown	Highly soluble
			Alkaline	Deep purple		
			Normal	Red		
SUFC S. undatus	Stable at ordinary light	Stable at room temperature	Acidic	Blue	Red to yellow	Highly soluble
			Alkaline	Yellow		
			Normal	Red		

S. No	Compound	PDB ID
1	Cyclooxygenase – 2 [Cox-2]	3MDL

2	Dipeptidyl peptidase – 4 [DPP-4]	1J2E
3	Superoxide dismutase [SOD]	1IDS

Table 10. The molecular docking studies of the phytochemical markers selected and their observed dock score

S. No	Compound	Enzyme	Amino acid resembles for hydrogen bond	H-bond distance	Affinity (kcal/mol)
1	Quercetin	COX-2	ASP229, ARG333, GLN241, SER146 and TRP139	9.751	-7.7
		DPP-4	ASN151, ASN169, ASN170, ASP192, THR129 and TRP201	0.000	-8.2
2	Gallic acid	SOD	ASN172, MET161, TYR36 and VAL120	12.861	-5.9
3	Celecoxib	COX-2	ASN375, ASN537 and GLN374	7.218	-8.3
4	Sitagliptin	DPP-4	GLN153, TRP201, TYR211 and TYR238	27.056	-7.5
5	Vit-C	SOD	GLU163, GLY122 and VAL120	1.670	-5.0

Phytochemical marker for C. sappan wood, C. ternata flowers: Quercetin. Reference standards used Celecoxib and Sitagliptin
Phytochemical marker for S. undatus fruits: gallic acid. Reference standard used Vit-C

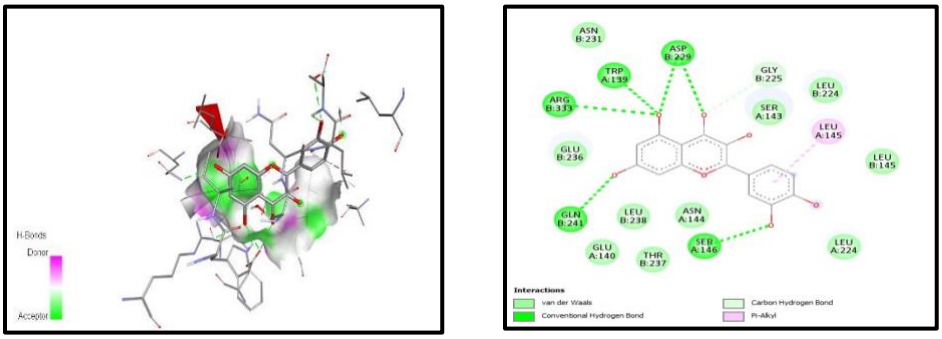
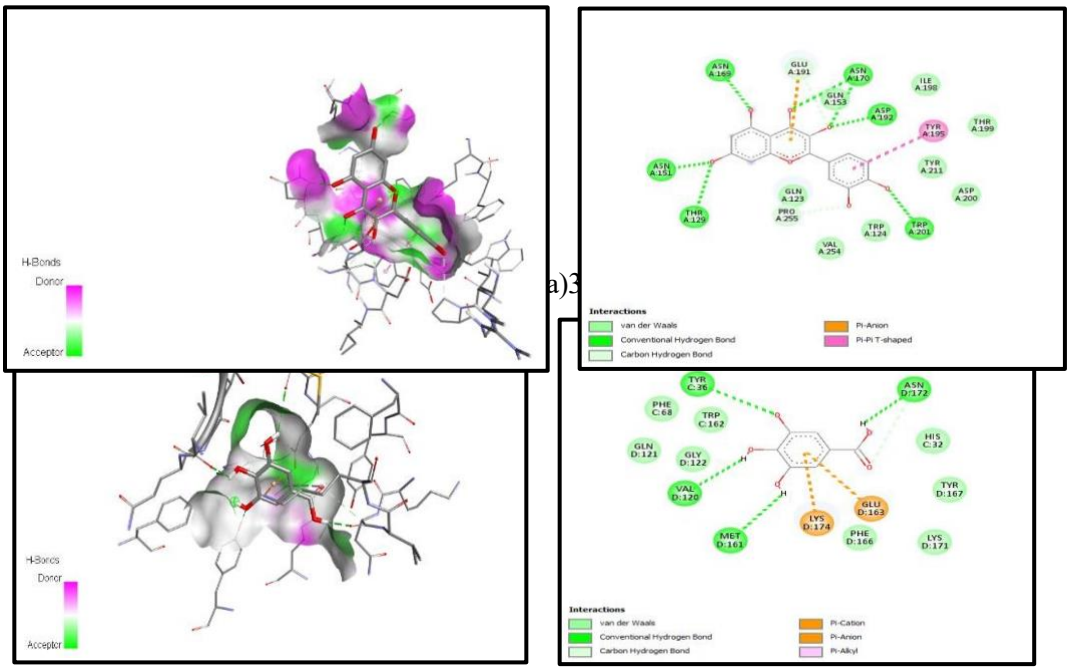


Fig.19. Ligand interaction diagram of COX-2 (a)3D and (b)2D poses of quercetin
Fig.20. Ligand interaction diagram of DPP-4 (a)3D and (b)2D poses of quercetin



RESULTS AND DISCUSSION

The colour extracts obtained from C. sappan wood, C. ternata flowers and S. undatus fruits were found to be highly soluble in water, therefore these colours can be incorporated easily in any pharmaceutical formulations like syrups, suspensions etc and also in food materials and nutraceuticals if they are suitably stabilised.

The yellow, blue and red colour variation of CSWC, CTFC and SUFC in acidic and alkaline pH can be of use to prepare many shades of colours from these pigments. The stability studies [26,27,28] indicated that all the extracted colours are highly soluble in water, stable at room temperature and ordinary light, but upon oxidation change the colour. All the colours react differently in acidic and basic medium with different shades of colours [19] Table.8. The percentage yield obtained for CSWC, CTFC, and SUFC are 90.5, 94.25 and 95.35 respectively.

The colour extracts CSWC, CTFC and SUFC showed an LC50 value of 7943.3, 6534.5 and 5623.4 mcg/ml respectively as compared with FCF color 15985,14720 (3981.07mcg/ml), Brilliant blue FCF (3771.7mcg/ml), Carmoisine (1989.57 mcg/ml) respectively in the brine shrimp assay method.

These values indicated that the natural colour pigments are much safer compared to their synthetic counterpart. According to Meyer's and Clarkson's toxicity index, extracts with LC50 < 1000 µg/ml are considered as toxic, while extracts with LC50 > 1000 µg/ml are considered as non-toxic [22,23]. Brine Shrimp Lethality utilising Artemia salina nauplii is found to be as one of the alternatives for the biological toxicity assays of herbal extracts, and this test turned out to be significantly correlated with several other animal models [25].

HPTLC evaluation of these colour extracts indicated the presence of the reference standard quercetin (R_f value 0.474, Toluene: Ethyl acetate: Formic acid [5:5:1]) in Clitoria flower and Sappan wood extracts (quercetin, R_f value 0.945, chloroform: methanol: water [64:50:10]) as 87.68 mcg/mg and 72.64 mcg/mg respectively. The amount of reference standard gallic acid [R_f value 0.45, Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.8:0.2)] in dragon fruit extract is found to be 38.46 mcg/mg.

The In-silico molecular docking studies revealed significant binding affinity for quercetin towards Cox-2 and DPP-4 enzymes (-7.7 and -8.2 kcal/mol) as compared to the reference standards Celecoxib and Sitagliptin (-8.3 and -7.5 kcal/mol) respectively. Gallic acid showed a binding affinity (-5.9) against the reference standard Vit C (-5.0).

CONCLUSION

The preliminary cytotoxicity data indicated that the colour extract CSWC, CTFC, and SUFC obtained from C. sappan wood, C. ternata flower petals, and S. undatus fruit are safe to use as a colouring agent in pharmaceutical and cosmeceuticals instead of synthetic FDC grade colours. Brine Shrimp Lethality Assay (BSLA) classifies as an in vivo toxicity testing method and the test is rapid, simple and requires minimum requirements. The BSLA test is an alternative toxicity assay, widely used for the testing of the toxicity potential of plant products, and the test considered significantly correlated with several other animal models.

The anti-inflammatory, antiulcer and astringent properties of this natural colour pigments, can be utilized in pharmaceuticals, cosmeceuticals and food materials not only as a colouring agent, but can impart many useful therapeutic activities. Clitoria ternatea blue color dye (has anti-diabetic action) if added to the formulation of anti-diabetic syrups, may produce additive effects, thereby the safety.

The red pigments found in dragon fruit help protect against bad cholesterol (LDL) by preventing its oxidation, also it has antioxidant property therefore if used in antioxidants syrups results in improved therapeutic action.

The percentage yield of CSWC, CTFC and SUFC were found satisfactory for sustainable commercial production. Also, the results showed that the routine analysis and validation of these three colour extracts can be done effectively by HPTLC techniques with therapeutic/analytical markers like quercetin (for sappan wood and Clitorea colours) and gallic acid (for dragon fruit colour).

In-silico molecular docking studies revealed that quercetin present in CSWC, CTFC and gallic acid present in SUFC showed significant binding affinity against COX-2, DPP-4 and SOD respectively.

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