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INSILICO MOLECULAR DOCKING AND DEVELOPMENT OF PIPER BETLE EXTRACT NANOEMULSION TO TREAT GOUT

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ABSTRACT

Gout is a pathological condition which is characterized by the uric acid in the form of monosodium Urate crystals get deposited in the joints, synovial fluid and other tissues and causes an inflammatory and painful condition called as gouty arthritis. The global gout incidence has increased by 63.44% over the past 2 decades. A computational ligand-target docking approach was used to analyze structural complexes of the target with bio active compounds (ligand) in order to understand the structural basis of this protein target specificity. Nanoemulsion was prepared by methanolic extract of *piper betle* leaves for efficient drug release for topical application in treatment of symptoms of Gout. The stable Nano-emulsion containing Piperol B, and quercitine and other constituents as active ingredient was successfully formulated the extract was prepared from leaves by soxhlation and used as active ingredient in Nano-emulsion. The activity of constituents toward the xanthine oxidase enzyme was determined by molecular docking.

KEYWORDS: Gout, Nanoemulsion, Docking score, *piper betle.*

INTRODUCTION

Gout is a pathological condition which is characterized by the uric acid in the form of monosodium Urate crystals get deposited in the joints, synovial fluid and other tissues and causes an inflammatory and painful condition called as gouty arthritis. Symptoms are Severe

pain, redness, Lumps, stiffness, swelling in joints often the big toe limited range of motion. Which is due to diet, genetic predisposition, under excretion of urate, salts of uric acid, insulin resistance, regular aspirin and niacin use.[1] Gout is a common and debilitating condition that is associated with significant morbidity and mortality. Despite advances in medical treatment, the global burden of gout continues to increase, particularly in high-sociodemographic index (SDI) regions.[2] Gout incidence in the young population grew simultaneously and substantially in both developed and developing countries.[3]

The global gout incidence has increased by 63.44% over the past 2 decades, with a corresponding increase of 51.12% in global years lived with disability. In both sexes the global gout incidence increased over time.[3]

Betel vine (*Piper betle L.*) belongs to genus Piper of the family Piperaceae. Piper betle leaves posses several bioactivity and are used in traditional medicine. Many research studies on Piper betle has reported that it contains important chemical constituents and are acts to arouse action for its medicinal properties like anticancer, anti-allergic, antimalaria, anti-filarial, antibacterial, antifungal study, insecticidal, antioxidant, anti-diabetic, gastro-protective, cytotoxic, wound healing activity, chlorophyllase activity, oral hygiene and anti-asthmatic effect.[4] *piper betle* contains numerous phytochemicals depending on its botanical origin and the solvent used for extraction. A preliminary phytochemical analysis of betel leaves shows the presence of alkaloids, tannins, glycosides, reducing sugars, and saponins in the water extract of betel leaves. The total content of phenol, flavonoid, and tannin was present in water, ethanol, ethyl acetate, acetone, and dichloromethane extracts of betel leaves.[5]

Fractionation and pure compounds from betel leaf extract has antioxidant activity. Besides, betel leaf extract also leads to increase antioxidant enzymes such as superoxide dismutase (SOD) and catalase Piper betle Linn methanolic extract demonstrates significant antioxidant activity of Piper betle Linn, which can be used as easily accessible source of natural antioxidant.[6]

Betle leaf extract contains active compounds hydroxyl chavicol (HC), Eugenol (EU), Chavibetal, allyl pyro catechol, Quercitin, estragole, Piperol-A, Piperol-B, methyl piper betlol. The betle leaves have starch, sugars, diastases and an essential oil composing of terpinen-4-ol, safrole, allyl pyrocatechol monoacetate, eugenol, eugenyl acetate, hydroxyl

chavicol, eugenol, piper betol and the betle oil contains cadinene carvacrol, allyl catechol, chavicol, p-cymene, caryophyllene, chavibetol, cineole, estragol.[7]

Nano emulsion: Thermodynamically or kinetically stable liquid dispersion of oil phase and a water phase in combination with a size range of 500nm-200nm. Nano emulsion due to small particle size of partial in these kinds of delivery systems(r<100nm) which enhance long term stability, high optical clarity and increased bio-availability. These Nano emulsion are prepared by the different methods like high pressure homogenization, ultra sonication. Phase inversion method, Micro fluidization.

Advantages of Nano emulsion than other dosage form: Increase rate of absorption, increase bio-availability, various routes like topical, oral, and intravenous can be made to deliver the product. Rapid and efficient penetration of the drug moiety, safe for administration due to absence of thickening agent and colloidal partials, required low amount of surfactant compared to micro emulsion Nano emulsion are thermo dynamically stable system and the stability allows self-emulsification of the system. Nano emulsion formulations required low amount of surfactant compared to micro emulsion.[8]

Computer-aided drug design (CADD) methodologies are playing an important role in drug discovery that are critical in the cost-effective identification of promising drug candidates. These computational methods are relevant in limiting the use of animal models in pharmacological research, for aiding the rational design of novel and safe drug candidates.[9]

A computational ligand-target docking approach was used to analyze structural complexes of the target with bio active compounds (ligand) in order to understand the structural basis of this protein target specificity.[10]

Thus in current study preparing nanoemulsion of the methanolic extract of *piper betel* leaves for efficient drug release by topical application in treatment of symptoms of Gout.

METHODS AND MATERIALS

Molecular docking

Material and Methods

ChemDraw software 20.1.1.125 (PerkinElmer Informatics, Inc., USA), OpenBabel 2.4.1. Software (Openeye scientific, New Mexico.), BIOVIA Discovery Studio 2020 v20.1.0.19295 (Dassault system, San Diego, CA, USA).

2.2.12. Molecular Docking Study

The two dimensional (2-D) structure all phytoconstituents like hydroxychavicol, chavicol, allypyrocatechol, estrgole, eugenol, piperol A, piperol B, methyleugenol, hydroxycatechol, piperbetol, methylpiperbetol were drawn using ChemDraw software 20.1.1.125 (PerkinElmer Informatics, Inc., USA) and saved in. cdx format. The interactions between all phyto constituents and protein TLR-2(PDB ID: 2Z80) http://www.japsonline.com was determined in silico by molecular docking using BIOVIA Discovery Studio 2020 v20.1.0.19295 software (Dassault system, San Diego, CA, USA). All drugs structures were converted to sdf MDL MOL format from. cdx file using OpenBabel 2.4.1. Software (Openeye scientific, New Mexico.) as single file. Ligand preparation was performed by minimizing energy for docking. TLR-2(PDB ID: 2Z80) was downloaded and prepared. The preparation of protein was based on selection of chain containing amino acids for respective co-crystal (2-acetamido-2-deoxy-beta-D-glucopyranose - NAG801) for chain A and water molecules were removed. After protein preparation binding site was defined with co-crystal. The prepared ligands were docked against prepared protein with CDOCKER inbuilt algorithm using BIOVIA Discovery Studio 2020. The interactions resulted in binding energy (kcal/mole), 2D and 3D interactions between respective ligand and protein Cite and protein Cite.[11]

3.12. Molecular Docking Analysis

The docking results were sorted for phyto constituents with least binding energy, maximum hydrogen bonding and minimum bond length. Quercitin and Piperol B were found to be having least binding energies. The interactions were visualized in 2D and 3D format included nature of bonding, bond length (Å) and respective binding energy in (kcal/mole) TABLE1followed by 3D images of interaction ***FIGURE1 AND FIGURE2.***

Table 1: Interaction results for docking studies for co-crystal NAG801-2-acetamido-2-deoxy-beta-D-glucopyranose, quercitin and piperol B.

Interaction	Binding Energy energy(kcal/mole)	Interacting amino acids and atoms	Nature of Bond	Bond length(Å)
2Z80- Co-crystal NAG801- 2-acetamido-2-deoxy-beta-D-glucopyranose	-67.0275	PRO/ A:135 and -OH of glucopyranose ring	Hydrogen bond	2.75
		ASN/A: 114 and -OH, acetamido of glucopyranose	Hydrogen bond	2.32, 4.62
		SER/ A:113 and two –OH at of glucopyranose ring	Hydrogen bond	2.65, 2.07
		LYS/ A:137 and –OH AT 3^{RD} and 4^{th}position of glucopyranose ring	Hydrogen bond	1.89

Table no: 02.

2Z80-Quercitin	-71.286	GLN/A:166 and -OH at 7th position	Hydrogen bond	5.08
		LYS/A: 137 and -CH_3 and -CH_3 at 17th and 18th position.	Alkyl bond	4.90 and 4.89
2Z80-Piperol B	-63.3551	THR/ A:138 and -C=O of oct-3-en-2-one	Hydrogen bond	2.33
		GLY/ A: 140 and oct-3-en-2-one, -O-CH_3 of and oct-3-en-2-one	Carbon hydrogen bond	2.58 and 2.38
		PRO/ A: 135 and hydrogen of 3,4-dimethoxyphenyl from, -O-CH_3	Carbon hydrogen bond	2.77
		SER/ A:113 and two hydrogen of 3,4-dimethoxyphenyl from, -O-CH_3	Carbon hydrogen bond	2.66 and 2.85
		LYS/A: 137 and 3, 4-dimethoxyphenyl ring, carbons of dimethoxy group.	Alkyl bond	4.02 and 4.94, 4.71

DISCUSSION

The 2D and 3D interactions of TLR-2(PDB ID: 2Z80) amino acids and functional groups of co crystal (shows 2Z80- Co-crystal NAG801- 2-acetamido-2-deoxy-beta-D-glucopyranose has the **Binding Energy of -67.0275(kcal/mole*) the i*nteracting amino acids and atoms were determined** PRO/ A:135 and -OH of glucopyranose ring the **nature of Bond was determined to be hydrogen bond of bond length 2.75 Å,** ASN/A: 114 and -OH, acetamido of glucopyranose found to have hydrogen bonding and bond length 2.32, 4.62 **Å**, SER/ A:113 and two –OH at of glucopyranose ring found to have hydrogen bond and the bond length 2.65, 2.07 **Å**, LYS/ A:137 and –OH of glucopyranose ring has hydrogen bond and the bond length 1.89 **Å**. ***Figure 1.***

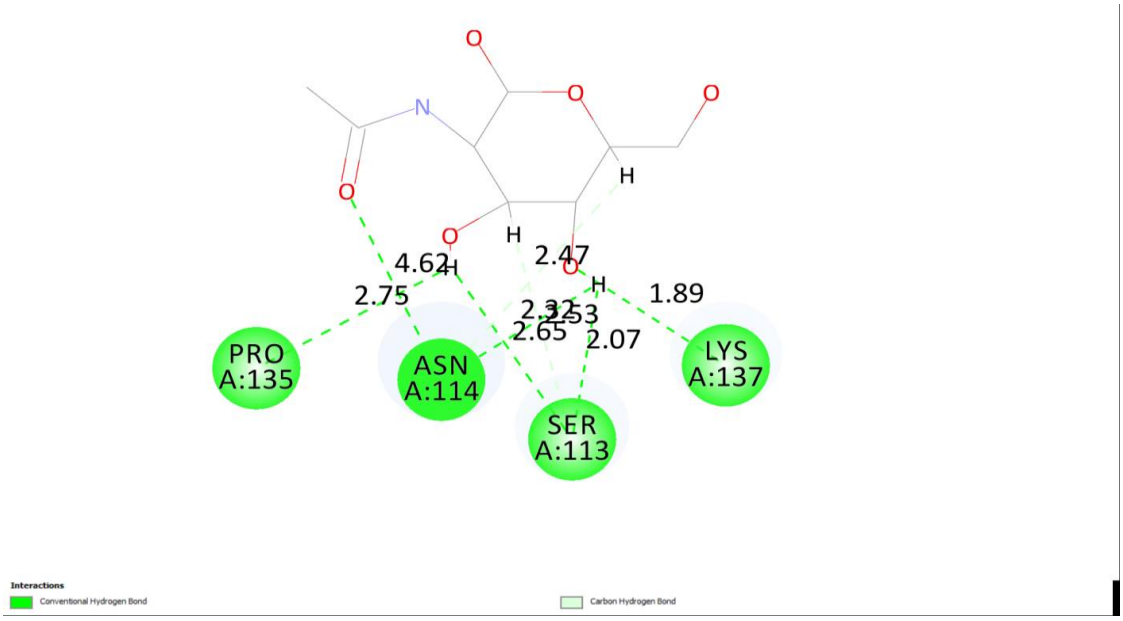
The 2D and 3D interactions of TLR-2(PDB ID: 2Z80) amino acids and functional groups of Quercitin shows 2Z80- Quercitin **has the binding energy of -71.286(kcal/mole) the interacting amino acids and atoms were determined** GLN/A: 166 and –OH has hydrogen bond and bond length of 5.08 **Å**, LYS/A: 137 and –CH3 and –CH3 has alkyl bond and bond length of 4.90 and 4.89 **Å**. ***Figure 2.***

The 2D and 3D interactions of TLR-2(PDB ID: 2Z80) amino acids and functional groups of Piperol B showed 2Z80-Piperol B has the binding energy -63.3551**(kcal/mole*) the* interacting amino acids and atoms were determined** THR/ A:138 and -C=O of oct-3-en-2-

one has hydrogen bond and bond length 2.33 **Å**,GLY/ A: 140 and oct-3-en-2-one, -O-CH_3 of and oct-3-en-2-one has carbon hydrogen bond. and bond length 2.58 and 2.38 **Å**, PRO/ A: 135 and hydrogen of 3,4-dimethoxyphenyl from, -O-CH_3 has carbon hydrogen bond and bond length 2.77 **Å,** SER/ A:113 and two hydrogen of 3,4-dimethoxyphenyl from, -O-CH_3 has carbon hydrogen bond and bond length 2.66 and 2.85 **Å**, LYS/A: 137 and 3,4-dimethoxyphenyl ring, carbons of dimethoxy groups has alkyl bond and bond length 4.02 and 4.94, 4.71 **Å**, ***Figure3.***

Compare to co-crystal the binding energy for Quercitin is less, hence has better affinity towards target than co-crystal. Similarly, Piperol B showed binding energy very close to co-crystal. Additionally, Piperol B has shorted bond length especially for carbon hydrogen bond which justifies better stability.

Table 1 Based on results, although Quercitin is showing least binding energy (prominent binding) the bond length observed between interacting amino acids and function groups is more compared to Piperol B. The larger bond distances may lead to unstable protein-ligand complex. Hence, we suggest Piperol B could be an appropriate selection for further studies.



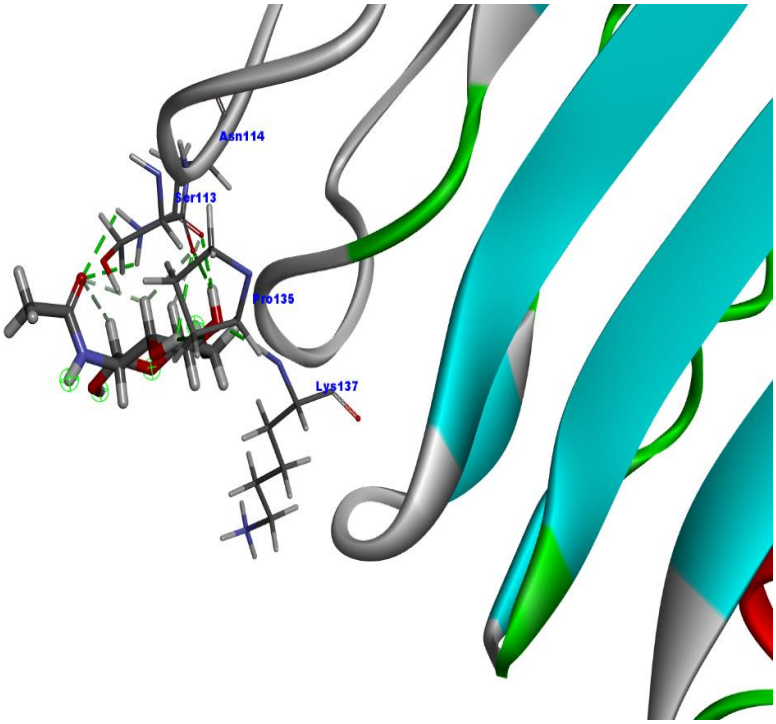
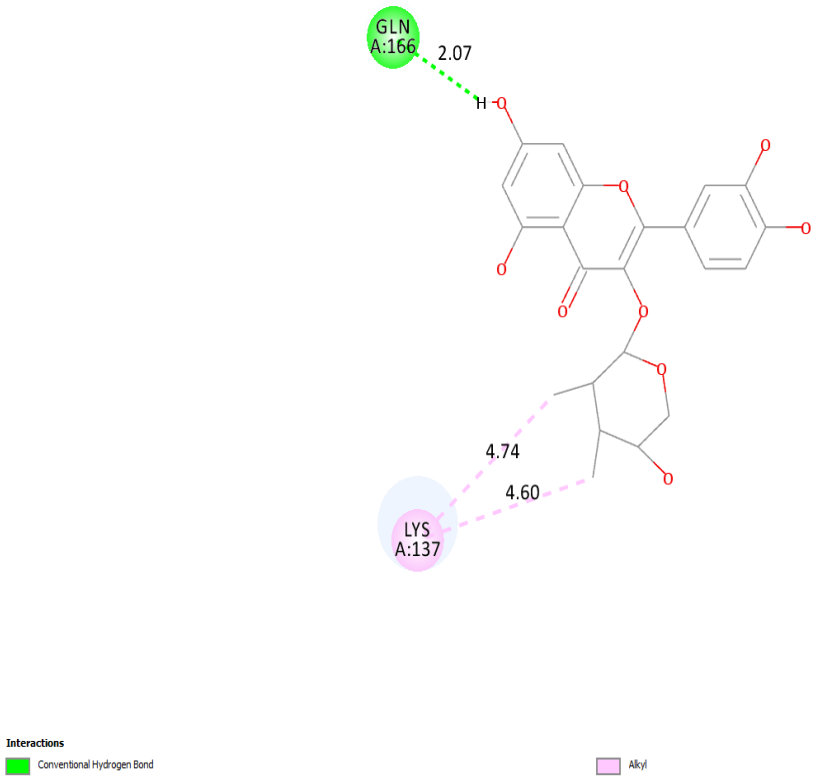


Figure 1: a) 2D and b) 3D interactions of TLR-2(PDB ID: 2Z80) amino acids and functional groups of Co-crystal 2-acetamido-2-deoxy-beta-D-glucopyranose (NAG801).



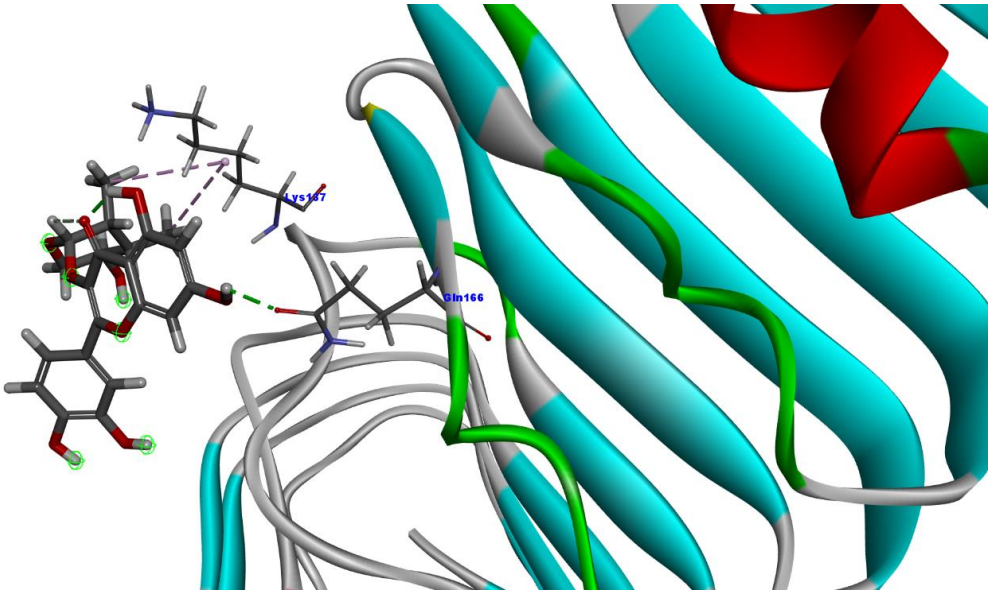
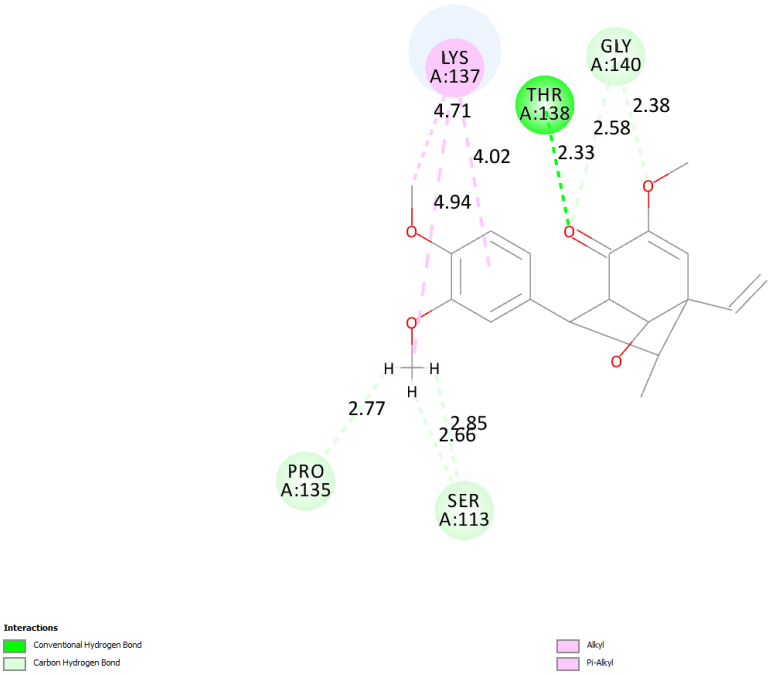


Figure 2: a) 2D and b) 3D interactions of TLR-2(PDB ID : 2Z80) amino acids and functional groups of Quercitin.



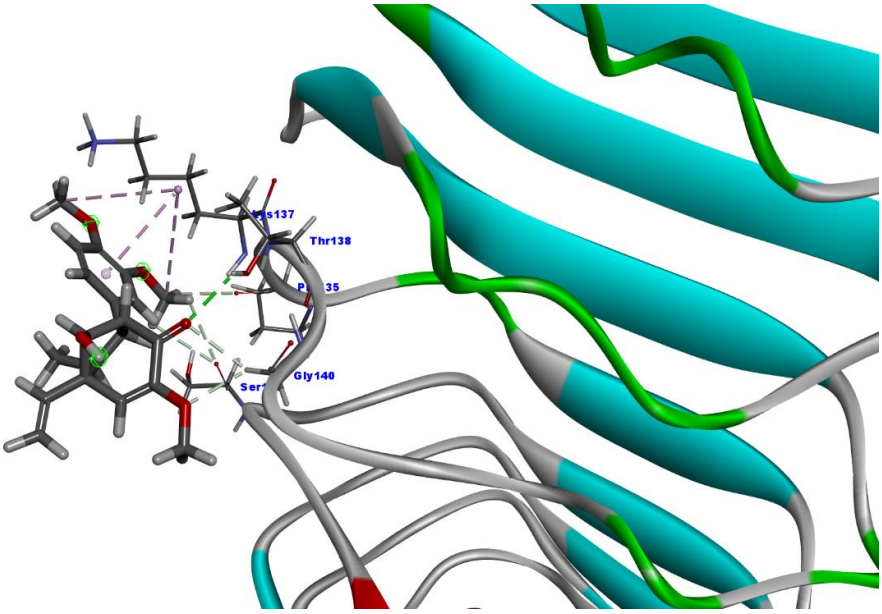


Figure 3: a) 2D and b) 3D interactions of TLR-2(PDB ID: 2Z80) amino acids and functional groups of Piperol B.

From insilico molecular docking studies piperol b shows good activity for treatment of gout. for the better efficient drug release nano emulsion of methanolic extract of piper betle was prepared.

Preparation of leaf extract

a.collection of Plant Materials Fresh leaves of Piper betle were collected from raghuvanahalli region, Bangalore, Karnataka. Leaves were shade dried and crushed into fine powder.

b. Preparation of Methanolic Extract- Dried and powdered leaves (25 g) were used. The extract is prepared using Soxhlet apparatus. Powdered leaves with 200 mL of methanol were subjected for extraction. The crude Soxhlet apparatus and concentrated to dryness in water bath. Concentrated extract was yielded, which is stored in refrigerator.

Pre-formulations

1. Determination of maximum absorbance wavelength

From the spectra maximum absorbance wavelength.

Standard Curve of extract in Acetone

100mg of extract was dissolved in 10ml of Acetone and made upto 100 ml with acetone, and further dilutions were made by using acetone to obtain concentrations ranging from 10, 20, 30,40,50,60,70,80,90 and 100µg/ml. The absorbance of solution was measured at 420nm and

520nm using UV –Visible Spectrophotometer. The readings obtained are tabulated in Table and graph.

CONCENTRATION	ABSORBANCE
10	0.13
20	0.21
30	0.27
40	0.32
50	0.41
60	0.53
70	0.58
80	0.69
90	1.98
100	1.38

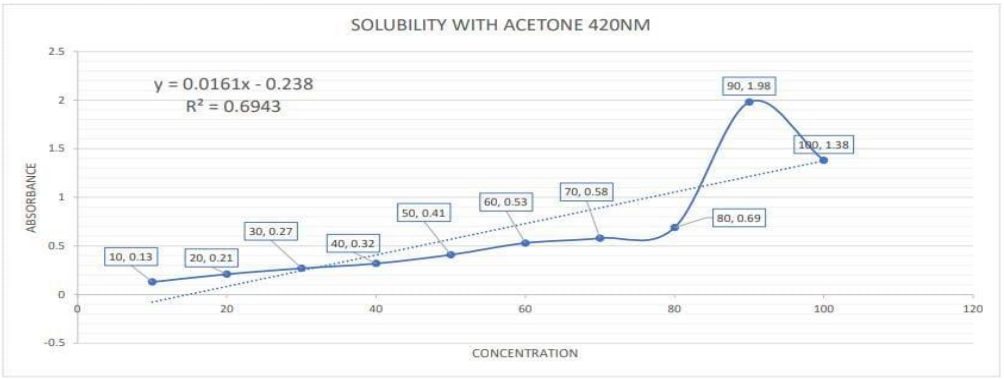


Figure no: 4.

CONCENTRATION	ABSORBANCE
10	0.08
20	0.1
30	0.12
40	0.11
50	0.17
60	0.28
70	0.29
80	0.38
90	1.19
100	0.54

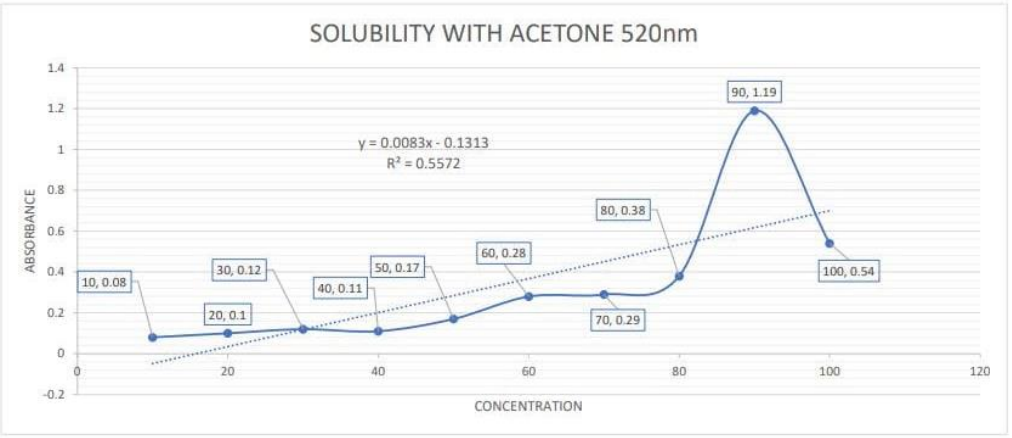


Figure no: 05

Standard Curve of poly herbal extract in Phosphate Buffer PH 7.4

100mg of polyherbal extract was dissolved in 10ml of Acetone and made up to 100 ml with phosphate buffer p^H 7.4 and further dilutions were made by using phosphate buffer p^H 7.4 to obtain concentrations ranging 10,20,30,4050,60,70,80,90 and 100µg/ml.. The absorbance of solution was measured at 420nm and 520nm using UV –Visible Spectrophotometer. The readings obtained are tabulated in Table and graph.

CONCENTRATION	ABSORBANCE
10	0.21
20	0.3
30	0.42
40	0.52
50	0.61
60	0.6
70	0.71
80	0.72
90	0.83
100	1.03

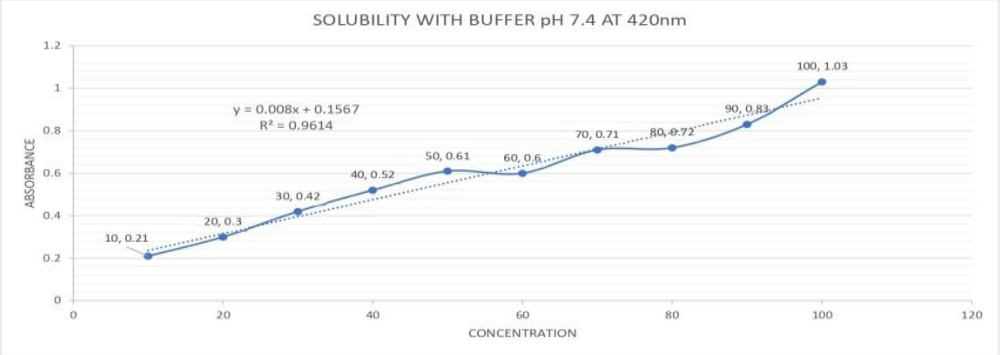


Figure no: 06

CONCENTRATION	ABSORBANCE
10	-0.03
20	0.01
30	0.05
40	0.09
50	0.17
60	0.21
70	0.26
80	0.29
90	0.34
100	0.43

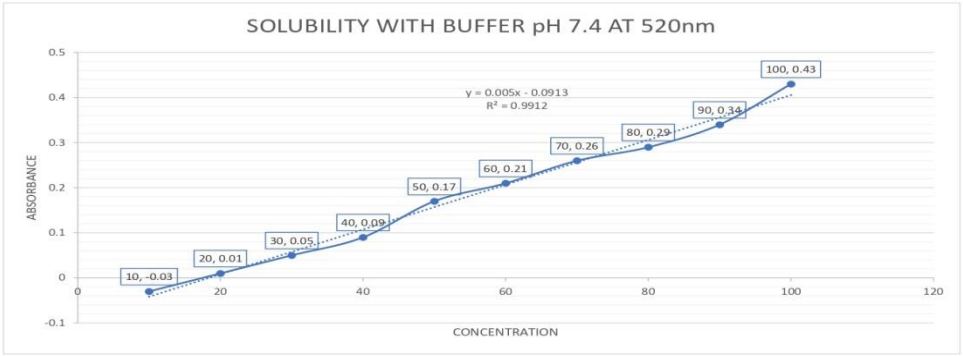


Figure no:07

From the standard curve of acetone and buffer PH 7.4 at 420 nm and 520nm. Buffer PH 7.4 showing good solubility for extract.

Saturation Solubility Studies: 10ml of different components are taken in 100ml conical flask separately and excess amount of the drug is added to the flasksupernatant is quantified by UV-VISIBLE spectroscopy after appropriate dilutions.

Table no 02.

S.NO	COMPONENT (3^{rd} dilution, 1/1000)	Absorbance	Drug content in 1ml of component
1	Soya bean oil	0.2	10ug/ml
3	Sunflower oil	0.2	10ug/ml
4	Olive oil	0.1	10ug/ml
5	Jinjeli oil	0.01	10ug/ml
6	Isopropyl meristate	0.01	10ug/ml
7	Tween 80	0.1	10ug/ml
8	Glycerol	0.0	10ug/ml
10	Propylene glycol	0.1	10ug/ml
11	PEG-400	0.447	10ug/ml

Preparation of pseudo ternary phase diagram

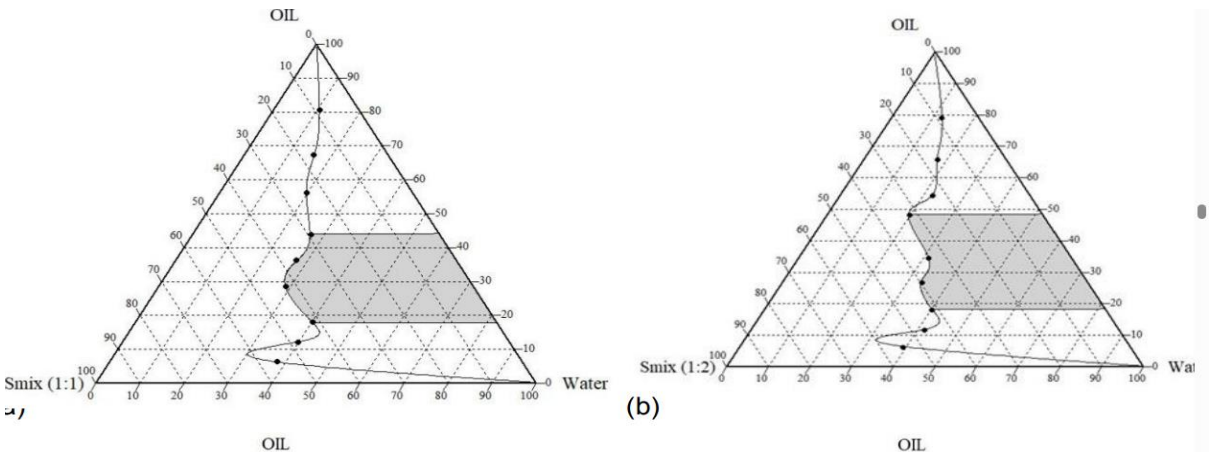
The ternary phase diagram between oil, S water is plotted to obtain the nanoemulsion region on the basis of results obtained in. On phase diagram an o/w Nano emulsion region was observed on high water ratio side. Beyond the Nano emulsion reason there was a phase separation and turbid mixture found for high and low oil concentrations respectively. The equal concentration of surfactant and co surfactant gave a good nanoemulsion reason (Fig. 2a), which further increased by increasing co-surfactant concentration to double of surfactant (Fig. 2b) but again increase in co surfactant concentration to triple of surfactant showed reduction in nanoemulsion region (Fig. 2c). So, no further concentration of co was increased. Similarly, the nanoemulsion region was also increase for in surfactant ration to double by co-surfactant (Fig. 2d) but again decreased for further increase (Fig. 2e). Therefore, there was no need to attempt more test with further increase in surfactant concentration. Fig. 1. Solubility of betel leaf extract in different oils, surfactants. solubility drug i.e., 38.6±0.091 Preparation of pseudo ternary phase The ternary phase diagram between oil, Smix and water is plotted to obtain the Nano emulsion region on the basis of results obtained in Table On phase diagram an o/w Nano emulsion region was observed on high water ratio side. Beyond the Nano emulsion reason there was a bid mixture found for high and low oil concentrations respectively. The equal concentration of surfactant and co-surfactant gave a good Nano

emulsion reason (Fig. 2a), which further increased by increasing surfactant concentration to double of (Fig. 2b) but again increase in co-surfactant concentration to triple of surfactant showed reduction in Nano emulsion region (Fig. 2c). So, no further concentration of co-surfactant was increased. Similarly, the Nano emulsion region was also increase for increasing the surfactant (Fig. 2d) but again decreased for further increase (Fig. 2e). Therefore, there was no need to attempt more test with further increase in surfactant.[12]

Table no 03: Nano emulsion formulations by different combinations of Oil, S Smix.

Sl No.	Smix Ratio	Formulation No	Drug (% w/v)	Oil	Smix	Water
1.	1:1	F1	1	43.80	29.20	27.01
2.	1:1	F2	1	36.23	36.23	27.54
3.	1:1	F3	1	28.7	42.55	29.08
4.	1:1	F4	1	17.86	41.67	40.48
5.	1:2	F5	1	48.00	32.00	20.00
6.	1:2	F6	1	34.25	34.25	31.51
7.	1:2	F7	1	26.49	39.74	33.77
8.	1:2	F8	1	17.86	41.67	40.48
9.	1:3	F9	1	44.44	29.63	25.93
10.	1:3	F10	1	33.56	33.56	32.89
11.	1:3	F11	1	25.32	37.97	36.71
12.	1:3	F12	1	17.05	39.77	43.18
13	2:1	F13	1	48.39	32.88	19.35
14	2:1	F14	1	37.88	37.88	24.24
15	2:1	F15	1	29.63	44.44	25.93
16	2:1	F16	1	19.23	44.87	35.90
17	3:1	F17	1	48.00	32.00	20.00
18	3:1	F18	1	39.68	39.68	20.63
19	3:1	F19	1	28.99	43.48	27.54
20	3:1	F20	1	20.55	47.95	31.51

%=Percentage; w/v= weight per volume; Smix= Surfactant: Co-surfactant; F-betle formulation.



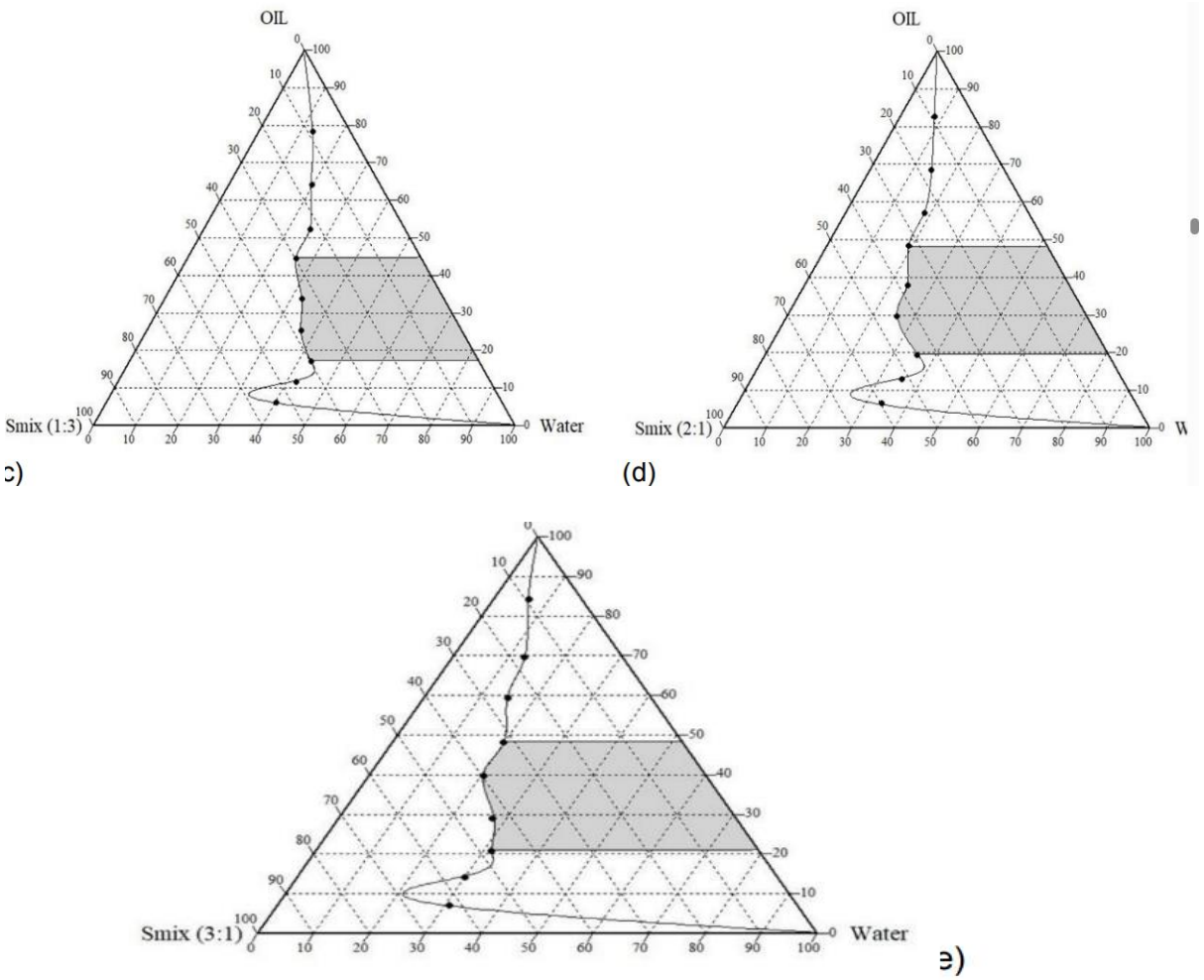


Figure no. 08.

In-vitro drug release studies

The results of in-vitro drug release profile of all eight selected formulations was observed that initially within 6 hours the release of drug from all formulations were very high and ranged between 75.20 ± 0.15 95.77 ± 0.14 %. After that from 6 to 12 hours the release rate was slightly decreased and 88.33 ± 0.64 % to 98.53 ± 0.36 %. After 24 hrs the range of release rate of formulations was found between 98.51 ± 0.25 % to 99.82 ± 0.28 % which is a very good release rate of drug from nanoemulsion. Formulations having top four release statics had been taken.

Particle size, polydispersity index and zeta potential

Table-5 shows the results of particle polydispersity index and zeta potential values of top four formulations having best drug release percentage. It was observed that all the four

nanoemulsion formulations had nano sized droplets. The PDI value of all these an 0.5 which indicated that each formulation has droplets spread uniformity. Also, the zetapotential of 28.8 to -12.4 which showed good repulsion force between nanoparticles results a good stability property of sin aqueous phase.

Table no. 04.

Sl. No	Formulation	Viscosity (cps) ± SD	pH ± SD	Refractive Index	Transmittance (%)	Absorbance
1	**F4**	6.946 ± 0.072	6.567 ± 0.0111	1.399 ± 0.007	95.29 ± 0.8	0.021 ± 0.0036
2	**F6**	9.114 ± 0.027	6.652 ± 0.019	1.41 ± 0.005	94.53 ± 1.4	0.0244 ± 0.0064
3	**F7**	8.856 ± 0.018	6.594 ± 0.014	1.407 ± 0.008	94.73 ± 0.4	0.0235 ± 0.0018
4	**F8**	6.946 ± 0.032	**6.567 ± 0.021**	1.398 ± 0.02	95.32 ± 0.7	0.0208 ± 0.0032
5	**F10**	8.526 ± 0.049	6.656 ± 0.017	1.408 ± 0.009	94.66 ± 1.1	0.0238 ± 0.0050
6	**F11**	7.695 ± 0.041	6.606 ± 0.013	1.403 ± 0.005	95.01 ± 1.5	0.0222 ± 0.0069
7	**F12**	6.149 ± 0.084	6.579 ± 0.022	1.395 ± 0.003	95.58 ± 1.2	0.0196 ± 0.0055
8	**F16**	8.631 ±0.094	6.546 ± 0.018	1.406 ± 0.006	94.84 ± 0.3	0.023 ± 0.0014

SD=Standard Deviation; %=Percentage; Cps=Centi poise; Values are mean ± SD; (n=40); ($p < 0.001$)

Table no, 05: Particle size, polydispersity index and zeta potential of Nano emulsion.

S. No	Formulation code	Droplet Size (d.nm)	Polydispersity Index	Zeta Potential (mV)
1	F6	21.9	0.345	-12.4
2	F7	21.5	0.334	-27.0
3	F10	19.5	0.342	-13.1
4	F16	18.9	0.363	-28.8

Transmission Electron Microscopy: In order to further confirm the Nano-droplet formation of formulations, the morphology of the best selected NE formulation (F16) was determined by TEM. The size distribution analysis in figure shows the uniformity of particles size.

CONCLUSION

The stable Nano-emulsion containing Piperol B, and quercitine and other constituents as active ingredient was successfully formulated the extract was prepared from leaves by soxhlation and used as active ingredient in Nano-emulsion. The activity of constituents toward the xanthine oxidase enzyme was determined by molecular docking. The docking results were sorted for phyto-constituents with least binding energy, maximum hydrogen bonding and minimum bond length. Quercitin and Piperol B were found to be having least binding energies. The interactions were visualized in 2D and 3D format included nature of bonding, bond length (Å) and respective binding energy in (kcal/mole). Although Quercitin is

showing least binding energy (prominent binding) the bond length observed between interacting amino acids and function groups is more compared to Piperol B. The larger bond distances may lead to unstable protein-ligand complex. Hence, we suggest Piperol B could be an appropriate selection for further studies. The surfactant and co-surfactant were selected based on the maximum absorption wavelength and the standard curves of extract in acetone and phosphate buffer PH 7.4 were obtained at 420 and 520nm and has shown good solubility for buffer at PH 7.4. Saturation solubility studies were conducted and pseudo-ternary phase diagrams were constructed and ratio of surfactant and co-surfactant using different oils. The Nano emulsion is prepared by adding the oils, surfactant, co-surfactant in suitable ratios (1:1, 1:2, 2:1,) Premixed emulsion is formulated to fix composition of the emulsion and then it was agitated at ultrasonic frequency of (20KHS) causing the droplet to break into Nano droplets by sonication method. Various evaluation studies were carried out like the viscosity of the poly herbal suspension was determined by using Brook-field viscometer. The diffusion studies were performed using egg membrane as a barrier layer 100ml of Phosphate buffer-7.4 was taken as a medium in the accept compartment.

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