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PREPARATIVE ISOLATION AND CHARACTERIZATION OF POLYPHENOLS FROM *ALPINIAGALANGA*LINN., *GARCINIA INDICA*CHOISY AND *GLYCYRRHIZAGLABRA*LINN., AND THEIR ROLE AS ANTI-OBESITY THERAPEUTICS RESEARCH PAPER

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ABSTRACT

Alpinia galanga plant which is associated with family Zingiberaceae is mainly scattered in tropical areas and widely known for ethno medicine. Against fungi and bacteria rhizome extract have a maximum inhibitory effect. *Alpinia galanga* plant is used in medicine and in food preparation. Rhizome extract of *Alpinia galanga* have high phenolic and flavonoid contents when compared to leaf extract. Because of elevated phenolic and flavonoid content in rhizome extract of *Alpinia galanga* there is noticeable antimicrobial as well as radical scavenging potential. It is a well-known official drug thought out the country as integrated contribution of nature. It is commonly used for the management of eczema, coryza, bronchitis, otitis interna, gastritis, ulcers, morbilli and cholera, pityriasis versicolor, to clear the mouth, emaciation. The different parts of the plant have various effects like antifungal, antiprotozoal, antiplatelet, antiviral, antidiabetic, immunomodulatory, antibacterial, anti-oxidant effects, hypolipidemic and many others. The essential oil of *A. galanga* identified 1, 8-cineol as a bioactive agent having antifeeding activity. An aqueous acetone extract of fruit of *Alpinia galanga* shows inhibitory effect on melanogenesis (formation of melanin). By using different methods, active constituent namely, 1'- acetochavicol acetate in hexane extract of *Alpinia galanga* rhizome was investigated for their corrosion inhibition properties. The current review add significant information about its, pharmacological activities, medicinal properties and phytochemical investigations as a traditional drug to cure for a number of diseases. Every fraction of the plant has valuable properties that can deliver humanity. The complete plant will be broadly investigated for further future prospective

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INTRODUCTION

Obesity is a new specialty of medicine fighting for recognition and a common nutritional disorder which is rapidly increasing in the past few decades for worldwide population. Obesity is an important serious health problem of the 21st century. Obesity has now been termed as a disease by the American Medical Association in 2013. Accordingly, the World Health Organisation (WHO) has described obesity as a global epidemic (Kyle et al., 2016). Obesity is a well-known health issue of the Western society and is on the rise in India (Kalra and Unnikrishnan, 2012).

ROLE OF PHARMACOTHERAPY IN OBESITY MANAGMENT

Mechanism of action for Anti-obesity allopathic drugs

Allopathic drugs like fenfluramine and lorcaserin demonstrates their anti-obesity effect by promoting 5-hydroxytryptamine, serotonin release. Both of these drugs are 5-HT receptor agonists. However, both drugs have been withdrawn from the market due to cardiac side effects (Rodgers et al., 2012, Halford and Harrold, 2012). Another drug, Rimonabant is a selective reverse agonist of the cannabinoid receptor type 1 (CB1) which works by increasing the differentiation of pre-adipocytes and synthesizing more triacylglycerol. However, this too suffered from some neuro and cardio metabolic risks and withdrawn from the market (Despres,2009).

PANCREATIC LIPASE AS TARGET FOR ANTI-OBESITY THERAPEUTICS

Pancreatic lipase inhibitors from plants represent the class of polyphenols having potential of natural anti-obesity substances. In obesity or *Medoroga*, *Meda*word stands for digestive process and *Medoroga*means disease related with the digestive problem or metabolism. Obesity is regarded as a disorder of lipid metabolism and that's why enzymes involved in this process are selectively targeted to develop anti- obesity drugs. Recently, newer approaches for the treatment of obesity have involved inhibition of dietary triglyceride absorption via inhibition of pancreatic lipase as this is the major source of excesscalories.

CHEMICAL CONSTITUENTS

The plant *A. galangal* has been studied widely by many researchers and a large number of chemical constituents have been isolated. 1′S-1′-acetoxychavicolacetate (ACE) is the major phenolic compound isolated from *A. galangal* (Yasuhara et al., 2009).Nineknownphenylpropanoidslike1′S-1′-acetoxychavicolacetateand1′S-1′- acetoxyeugenol acetate have been isolated from its rhizomes (Matsuda et al., 2003b). Other two constituents like p-hydroxycinnamaldehyde and [di-(*p*-hydroxy-cis-styryl)] methane have been reported from the chloroform extract of *A. galanga*rhizome (Barik et al., 1987). The pungency of *A. galanga*rhizomes is due to the presence of 1′-acetoxychavicolacetate also known as galangal acetate (Yang and Eilerman, 1999). Several other phenylpropanoids have been isolated from the plant (Zhu et al., 2009a, 2009b).

CHEMICAL CONSTITUENTS

Phytochemical composition of different parts of Kokum tree Fruit rind

Phytochemical studies revealed that fruit rind of *G. indica* contains moisture (80 %), protein (1 %), tannin (1.7 %), pectin (0.9 %), total sugars (4.1 %), crude fat (1.4 %), Organic acid as hydroxyl citric acid (5.9 %) and pigment (2.4 %) (Krishnamurthy et al., 1982).

Leaves

Kokum leaves have been reported to contain moisture (75 %), protein (2.3 %), fat (0.5 %), fiber (1.24 %), carbohydrates (17.2 %) and minerals like calcium (0.25 %) and iron (0.15 %). It is also reported to contain ascorbic acid (0.01 %) and oxalic acid (0.18 %) (Baliga et al., 2011).

Seed

The seeds of Kokum are considered to be rich source of fatty acid triglycerides mainly stearic and oleic acids (Baliga et al., 2011). Kokum seed also contains 25 % edible yellow colored fat, known as kokum butter, extracted from crushed seeds after boiling them in water and removing the fat from top or by churning the seeds in water. Free fatty acids constitute around 7.2 % of total kokum butter. This kokum butter has much more significance in cosmetic industries where it is widely used for preparation of lotions, creams, lip-balms and soaps (Jagtap et al., 2015).

Phytochemicals isolated from Kokum tree

Hexane extract of *G. indica*fruits yields one polyisoprenylatedbenzophenone and a new acylphloroglucinol derivative with another two therapeutically important molecules such as garcinol and isogarcinol (Kaur et al., 2012). Two isoprenylatedbenzophenones, xanthochymol and isoxanthochymol are present in the extracts of fruit rinds, stem bark, seed pericarps and leaves of *G. indica.* Xanthochymol and isoxanthochymol belongs to prenylatedbenzophenone class of compounds. Both xanthochymol and isoxanthochymol have been isolated from methanolic extract of the fruit rinds of *G. indica*(Chattopadhyay and Kumar, 2006).

PHARMACOLOGICAL ACTIVITIES ANTIOXIDANT ACTIVITIES

Antioxidants nullify the deleterious effects of reactive oxygen and nitrogen species. Plants rich in polyphenols like flavonoids, phenolic acids, terpenes, anthocyanins etc. have been reported as effective free radicals scavengers (Fraga et al., 2019). Studies have been reported that chloroform extract of Kokum rinds possess antioxidant properties in β-carotene-linoleate and DPPH assays *in vitro* (Selvi et al., 2003). Aqueous and ethanolic extracts of *G. indica*fruits are reported for antioxidant properties *in vitro* in both DPPH and superoxide anion models. It is also reported to reduce the levels of LPS-induced intracellular reactive oxygen species. Garcinol is oneofthemedicinallyimportantchemicalfrom*G.indica*whichpossessesseveral pharmacological activities. It suppresses protein glycation in a bovine serum albumin/fructose system and is one of the effective antioxidants and glycation inhibitor (Yamaguchi et al., 2000a).

Anthocyanins from *G. indica*have been described effective agents against lipid peroxidation in comparison to alpha-tocopherol. The anthocyanins are also reported to contain oxidative enzymes and exhibition of cardioprotective action. Bioflavonoids from *G. indica*such as leucoanthocyanidins, catechin, flavonols have been reported to improve permeability and strength of capillaries (Jagtap et al., 2015).

ANTIDIABETIC ACTIVITIES

Kokum has been traditionally used as anti-diabetic drug. Garcinol has been found to lower fasting blood glucose levels in streptozotocin induced hyperglycemic rats in acute study (Mali et al., 2017). Kokum has also restored the levels of erythrocyte GSH, an intracellular antioxidant which prevents the risk of developing secondary complications of hyperglycemia (Kirana and Srinivasan, 2010).

ANTI-INFLAMMATORY ACTIVITIES

Aqueous and ethanolic extracts from Kokum rind exhibited anti-inflammatory potential, showing powerful reduction in inflammation against carrageenan-induced paw edema model in an acute study. These extracts are also reported to show significant reduction in lysosomal enzymes like acid phosphatase and alkaline phosphatase (Khatib et al., 2010).

HEPATOPROTECTIVE ACTIVITY

Panda and co-workers, 2012 have reported antioxidant and hepatoprotective effect of fruit rind of *G. indica*in ethanol-induced hepatic damage rodents (Panda et al., 2012).

CARDIOPROTECTIVE ACTIVITIES

Cardiovascular diseases usually result in cardiac fibrosis which leads to thickening and stiffening of cardiac muscle progressing to heart failure. Garcinol inhibits acetyl transferase p300/CBP, which stops excessive expression of collagen-1 in cardiac fibroblasts in fibrotic condition (Chan et al., 2010). Cyanidin-3-glucoside isolated from Kokum enhances eNOS expression and increases NO production and thus exhibited improved endothelial dysfunction, harmonized blood pressure and effectiveinatherosclerosis(Xuetal.,2004).

NTIULCER ACTIVITIES

The incidence of peptic ulcer has been rising globally. Garcinol is also effective as gastroprotective agent in the treatment of gastric ulcers caused by chronic infection with *Helicobacter pylori* (Kaur et al., 2012). The oral administration of garcinol (40–200 mg/kg) provides anti-ulcer effects against indomethacin- induced gastric ulcerations in rats. The gastroprotective effects of garcinol at 200 mg/kg has been reported to be far better than that of cetraxate-HCl used as a positive control (Yamaguchi et al., 2000b).

NEUROPROTECTIVE ACTIVITIES

Methanolic extract of Kokum fruit exhibits significant neuroprotective potential against 6-OHDA, and found effective in anti-Parkinson's study in rats (Antala et al., 2012). Garcinol from *G. indica*reported as a neuroprotective agent acts by reducing the expression of LPS-induced inflammatory mediators, iNOS and COX-2 and prevents nitric oxide accumulation in LPS-treated astrocytes. Another chemical, cyanidin-3-glucoside present in Kokum has been reported to prevent the neurite outgrowth and the expression of neurofilament proteins and demonstrated neuroprotective potential (Chen et al., 2009).

ANTI-BACTERIAL AND ANTI-FUNGAL ACTIVITIES

Kokum leaf extract documented to possess inhibitory activity against certain pathogenic bacteria like *Salmonella typhi, S. parartyphi A* and *S. tymphimurium.* Similarly, aqueous extract of Kokum rind reported highest antibacterial activity against *Bacillus subtilis*, followed by *Escherichia coli, Enterobacteraerogenes*and *Staphylococcus aureus.*

Compound GG2

Compound **GG2** was obtained as white coloured waxy mass from hexane-ethyl acetate (80:20 *v/v*) eluents. Its FTIR spectrum absorption bands for hydroxyl group (3391 cm^{-1}) and unsaturation (1594, 1384 cm^{-1}). On the basis of ^{13}C NMR and mass spectrum, the molecular weight of **GG2** was determined to be 414 corresponding to the molecular formula $C_{29}H_{50}O$. The formula indicated the presence of five double bond equivalents; four of which were adjusted in a tetracyclic steroidal moiety and the remaining one in a vinyliclinkage.

^{1}H NMR spectrum of **GG2** exhibited a downfield signal at 5.34 assigned to H-6 vinylic proton. Another one-proton broad multiplet at 3.52 was ascribed to H-3 cabinol proton. The methylene and methine protons of the compound resonated between 2.28 and 1.01. The spectrum also displayed three doublets at 0.92, 0.78 and 0.69 (*J* = 8.0 Hz and each integrating for three protons) that were attributed to the protons associated with secondary methyl groups Me-27, Me-29 and Me-26, respectively. The protons of the two tertiary methyl groups Me-18 and Me-19 resonated as two three-proton singlets at 0.84 and 1.00, respectively.

^{13}C NMR spectrum of **GG2** exhibited signals for 29 carbons, importantly for vinylic carbons at 141.2 (C-5) and 122.4 (C-6); carbinol carbon at 72.4 (C-3) and six methyl carbons between 29.2 and 12.0. On comparison to the earlier reports, the structure of **GG2** was found to be stigmast-5-en-3-ol (sitosterol).

Compound GG3

Compound **GG3**, designated as lupenoic acid, was obtained as brown coloured sticky mass from hexane-ethyl acetate (75:25 *v/v*) eluents. Its FTIR spectrum displayed absorption bands at 3341 cm^{1} (O-H stretching), and 1755 cm^{-1} (C=O streching) and 1636 cm^{-1} (C=C stretching). Based on ^{13}C NMR and mass spectra, the molecular weight of **GG3** was found to be 438 consistent with the molecular formula $C_{30}H_{46}O_2$. Its +ve MS displayed a pseudo-molecular ion peak and a fragment ion peak arising due the loss of COOH with 2H from the parent peak at *m/z* 439 and 391 supporting the presence of a carboxylic group in **GG3**. The -ve MS exhibited a molecular ion peak at *m/z* 438 and fragment ion peaks arising due to $C_{12/13}$-$C_{8/14}$ fission at *m/z* 235 $[C_{17}\ H_{31}]^-$ and 203 $[C_{13}H_{15}O_2]^-$ along with base peaks at 151 that further supported the presence of a lupene type triterpene with a carboxylic group at the junction of rings D andE.

^{1}H NMR spectrum of **GG3** displayed two downfield broad doublets at 5.56 and 5.36, each integrating for one-proton, assigned to H-12 and H-11 vinylic protons, respectively. The spectrum also exhibited two one-proton singlets at 4.73 and 4.60 typical for the exocyclic protons H-29 a and H-29 b of the lupene triterpenes. The protons asociated with the six tertiary methyl groups resonated as six three-proton singlets between 1.04 and0.75.

^{13}C NMR spectrum of **GG3** exhibited signals for 30 carbons. The important signals appearedforcarboxyliccarbonat 179.5(C-28);vinyliccarbonswithintheringat 129.4(C-11)and130.2(C-12);andexocyclicvinyliccarbonsat 151.4(C-20)and111.2 (C-29). The NMR data of **GG3** was compared with other triterpenes (Mahato and Kundu, 1994) and found to be in good agreement with the lupene-type triterpenes. On this basis, the structure of **GG3** was established as lup-11, 20(29)- dien-28-oicacid.

Compound GG4

Compound **GG4** was obtained as yellow coloured crystals from ethyl acetate methanol (95:5 v/v) eluents. Its FTIR spectrum exhibited characteristic absorption bands for hydroxyl group (3405 cm^{-1}), C=C conjugation (1595 cm^{-1}) and ether moiety (1072 cm^{-1}). Based on ^{13}C NMR and mass spectra, the molecular weight of **GG4** was determinedtobeestablishedas164correspondingtothemolecularformula$C_{10}H_{12}O_2$. The formula indicated the presence of five double bond equivalents, four of which were adjusted in an aromatic ring and one in a vinylic bond. The mass spectrum of **GG4** also exhibited diagnostic peak at *m/z* 150 and 148 due to loss of methyl group and at *m/z* 120 and 132 due to the loss of CH_2OCH_3 and methoxy groups from parent ions respectively when recorded in +ve and –vemodes.

^{1}H NMR spectrum of **GG4** displayed two double doublets at 4.89 (*J* = 10.5, 4.5 Hz) and 4.86 (10.5, 5.5 Hz), each integrating for two protons, assigned correspondingly to H-2 and H-5 and H3 & H-6 aromatic protons. The protons associated with the exocyclic vinylic linkage appeared as a downfield broad multiplet at 5.33 (H-8) and as a doublet at 4.22 (*J* = 8.0 Hz, H-7). High value for the coupling constant reflected the trans arrangement of these vinylic protons. The oxygenated methylene protons H_2-9 resonated as two one-proton signals at 4.44 (dd, *J* = 11.0, 5.5 Hz) and 3.65 (br m). The methoxy protons appeared as a three-proton singlet at 3.36.

^{13}C NMR spectrum of **GG4** exhibited twelve signals, consisting of one oxygenated aromatic signal at 153.6 (C-1); four aromatic methylene signals for C-2, C-3, C-5, C-6; one aromatic methine signal for C-4; two vinylic carbons at 130.5 (C-7) and127.7 (C-8); one oxygenated methylene carbon at 79.6 (C-9) and a methoxy carbon at 55.6.Based on above discussion, the structure of **GG4** was deduced to be coumaryl alcohol-γ-*O*-methyl ether. It is a well known phenylpropanoid earlier isolated from *Alpiniagalanga*(Nam et al., 2005) and found to be antioxidant and antiinflammatory in effect (Yu et al., 2009).

Compound GG5

Compound **GG5**, designated as glycyrcatechin, was obtained as yellowish white coloured crystalline powder from hexane-ethyl acetate (60:40 *v/v*) eluents. Its FTIR spectrum exhibited absorption bands for hydroxyl groups (3385 cm^{-1}) and aromatic moiety (1594 cm^{-1}). Based on ^{13}C NMR and mass spectra, the molecular weight of **GG5** was determined to be 284 consistent with molecular formula of $C_{16}H_{14}O_5$ of a substituted catechin.C NMR spectrum of **GG5** displayed signals for sixteen carbons. Five downfield signals at 175.0, 163.0, 159.4, 157.9 and 153.6 appeared due to oxygenated methinecabons C-3, C-7, C-9, C-4 and C-3, respectively. The remaining methylene aromatic carbons resonated between 130.5 and 102.6. It also displayed a diagnostic peak at 79.6 attributable to oxygenated methine C-2 of flavan moiety. Another upfield signal at 55.6 was assigned to methoxy carbon.

Based on the foregoing account, the structure of **GG5** was elucidated as 7-methoxy 2- (3′, 4′-dihydroxyphenyl)-2H-1-benzopyran-3-ol. This is a 3,4-didehydro flavan-3-ol derivative that belongs to the class of catechins. On comparison of data with other catechins, it was found to be different from dehydroglyasperin C and D (Seo et al., 2010; Kim et al., 2012) and has been designated as Glycyrcatechin.

Table 5.7: NMR chemical shift data of GG1.

Position	^{1}H NMR	^{13}C NMR
2	-	154.2
3	5.34 br s	130.4
4	-	180.1
5	6.79 d (9.5)	129.4
6	6.87 dd (9.5, 3.0)	108.4
7	-	152.3
8	5.37 br s	106.2
9	-	150.9
10	-	110.1
1′	-	128.9
2′	6.36 dd (8.0, 3.5)	114.8
3′	6.29 d (8.0)	117.8
4′	-	149.8
5′	5.62 d (10.0)	117.0
6′	5.56 dd (10.0, 4.0)	112.1
1″	4.36 br m	76.4
2″	1.79	34.0
3″	1.63	29.5
4″	1.54	30.4
5″	6.67 dd (10.0, 4.5)	129.9
6″	6.64 dd (10.0, 4.0)	121.8
7″	1.30	32.4
8″	1.29	28.5
9″	1.18	22.4
10″	0.89 (t, 6.5)	14.2

Coupling constants in Hertz are provided in parentheses. Overlapped signals are mentioned without multiplicities.

Table 5.8: NMR chemical shift data of GG4.

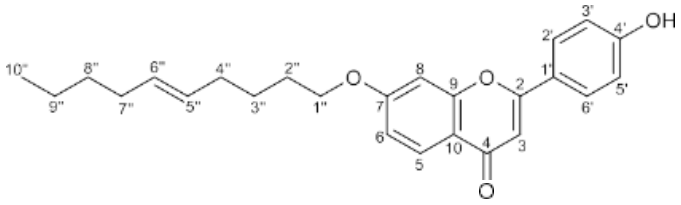
Position	^{1}H NMR	^{13}C NMR
1	-	153.6
2	4.89 dd (10.5, 4.5)	115.6
3	4.86 dd (10.5, 5.5)	102.6
4	-	117.0
5	4.89 dd (10.5, 4.5)	123.6
6	4.86 dd (10.5, 5.5)	114.0
7	4.22 d (8.0)	130.5
8	5.33 br m	127.7
9	4.44 dd (11.0, 5.5), 3.65 br m	79.6
OMe	3.36 s	55.6

Coupling constants in Hertz are provided in parentheses. Overlapped signals are mentioned without multiplicities.

Table 5.9: NMR chemical shift data of GG5.

Position	^{1}H NMR	^{13}C NMR
2	6.83 br s	79.6
3	-	175.0
4	8.32 s	130.5
5	6.97 d (8.5)	117.0
6	7.51 d (8.5)	114.0
7	-	163.0
8	7.96 br d	102.6
9	-	159.4
10	-	115.6
1′	-	127.7
2′	6.91 br d	124.7
3′	-	153.6
4′	-	157.9
5′	7.51 d (8.5)	123.6
6′	6.97 d (8.5)	114.0
OMe	3.32 s	55.6

Coupling constants in Hertz are provided in parentheses. Overlapped signals are mentioned without multiplicities.



IN SILICO DOCKINGSTUDIES

In silico approaches have been widely acknowledged to be useful for research and development and has been applied in herbal drug research as well. Because there are several thousand pharmacological targets and most natural compounds demonstrate pleiotropic effects by interacting with different targets, computational methods are the methods of choice in drug discovery based on natural products (Waghulde et al., 2018). *In-silico* computational docking modelling anticipate the most likely potential natural hits based on available protein data banks (PDR) to have interactions with residues of enzyme binding site and pharmacophore (Buchholz et al., 2014). Computational models generate useful predictions to be checked with further experimental results by using *in vitro* and *in vivo* models as per the requirement of particular research.

DOCKING STUDY

Representative polyphenols namely cyanidin, proanthocyanidin, flavanoyl flavone and the compounds isolated from *G. indica*were docked with pancreatic lipase enzyme to assess their interaction and binding modes with the target enzyme using Glide extra precision (XP) Maestro 10.1 Schrodinger, running on Linux 64 operating system (Schrodinger, Version 10.1, 2016). The enzyme PL is a validated target for anti-obesity drug and the crystal structure was downloaded from protein data bank (PDB 1LPB) (Sandeep et al., 2014). The protein preparation was done in Three steps mainly preprocess, review & modify and refinement were done for the protein preparation using protein preparation wizard. In these steps, water molecules are deleted and hydrogen atoms are added. The Energy of the structure was minimized using OPLS 2005 force field. Similarly, ligands were prepared again using force field 2005. Receptor grid generation program was run by clicking any atom of the ligand and the default box was prepared. The ligand was docked into the grid generated from the protein using extra precision- (XP). The results were evaluated by glide score (docking score) (Akhtar et al.,2017).

MM-GBSA free binding energy

Prime molecular mechanics-generalized born surface area (MM-GBSA) was calculated using Maestro 10.5. It is a tool to calculate ligand binding energy. A set of test compounds along with the standard Orlistat were used against the enzymepancreatic lipase (PDB ID -1LPB). Protein preparation and the ligand preparation were done from the above described methods. Alternatively the MM-GBSA results may be procured running the MM-GBSA program directly from the file generated by running the docking protocol (Siddiqui et al.,2016).

RESULTS AND DISCUSSION

Docking studies of representative polyphenols and compounds isolated from *G. indica*fruits

The docking study was carried out to know the binding mode of the representative polyphenols (cyanidin, proanthocyanidin and flavanoyl flavone) and the compounds isolated from *G. indica*fruits inside the PL receptor binding pocket. Orlistat was used as standard for comparison. The results were evaluated by glide score (docking score), higher the docking score more is the binding affinity. The docking score, binding free energy, and hydrogen bonds, pi-pi stacking formed with the surrounding amino acids are used to envisage their binding affinities and proper alignment of these compounds within the active site of pancreatic lipase.

All the test compounds (2D & 3D) showed hydrogen bond interaction with different residues and were compared with standard compounds (2D & 3D). The docking scores of the tested compounds and co-crystal ligand methoxyundecylphosphinic acid were presented in Table 6.1. Tested polyphenols showed hydrogen bond interaction with different residues and were compared with orlistat (Figure 6.1.A to 6.1.D). The results revealed that Proanthocyanidin is showing key hydrogen bond interaction with Asp 79, Phe77, Leu 153 and Ser152 similar to the hydrogen binding interaction shown by the standard drug Orlistat. Further the test drug proanthocyanidin shows additional hydrogen bond with Glu179, Tyr114 and which may be reasoned for more strong binding with the receptors. The docking score of the proanthoicyanidin was found to be -6.545 which is higher than the orlistat (-5.402). The free binding energy results from the Table 6.1 showed that all the compounds fit into the pancreatic lipase binding domain (PDB ID -1LPB), the best one among them is the compounds having more favorable conformation and showed highest DG binding (Kcal/mol). The binding energy was in the range of -36.49 to -49.95 Kcal/mol. The binding energy of the test compound proanthocyanidin was found to be -49.95 which is higher than the standarddrugOrlistat(-47.41Kcal/mol).Thehigherbindingenergyofthetestdrugshows stronger binding to the receptor than the standard drug Orlistat. Other two docked polyphenols, named cyanidin and flavanonyl flavone possessed low binding energy than proanthocyanidin and standard drug.

The isolated compounds were found to strongly inhibit PL by completely occupying the active sites in the target protein. All inhibitors showed good docking scores than the co-crystal ligand, taken as the standard. Among all the titled compounds for the receptor, oleantrienoic acid glucoside was found to be most potent and have high docking score of -10.11. This ligand also assumes favourable orientation within the PL-colipasecomplex binding site. The binding mode of oleantrienoic acid glucoside is exactly same as the co-crystal ligand. The 2D docked pose of oleantrienoic acid glucoside possesses two hydrogen bond interactions among which one of the hydroxyl group attached at 3-glycoside formed H-bond with a backbone residue PHE 77 and other 6-methylhydroxy of glycoside group formed a hydrogen-bond with a side chain residue HIE 151. Strong interaction with a backbone residue of oleantrienoic acid glucoside is the reason for its high affinity. All hydrogen- interactions of receptor- oleantrienoic acid glucoside complex in 3D-image is represented in Figure 6.1.E. The docked pose of second most dock ranked ligand luteolin (Docking score of -9.46), the hydroxyl group of dihydroxy chromen-4-one forms hydrogen-bond with both HIE 151 (side chain residue) and GLY 76 (backbone residue) as exhibited by oleantrienoic acid glucoside. One more similar pi-pi interaction was obtained by phenyl ring of dihydroxy phenyl ring at second position of luteolin with backbone residue PHE 77. Both rings of chromen-4-one forms pi- cation with HIP 263 which fills the compounds' account with penalties and hence it fell on second position with docking score less than oleantrienoic acid glucoside (Table 3). The interactions exhibited by the luteolin are shown in Figure 6.1.F. The third ligand napthyldioxolol possess only one hydrogen bond of hydroxyl group with SER 152 which is a sidechain residue and hence possesses least score of -8.19 among all three ligands. The 3D docked pose of napthyldioxolol is shown in Figure6.1.G.

The results related to the free binding energy showed that all the compounds fitted well in the PL binding domain (Table 6.1). The best one among them *i.e*., oleantrienoic acid glucoside has more suitable conformation to fit into the domain. The binding energy ranged between -35.000 and -44.021 Kcal/mol. The binding energy of the test compound luteolin was found to be-44.021 which is comparable to or list at (-47.41 Kcal/mol). Schematic diagrammatic representation are given in the Figures 6.1A-G

Table 6.1: *In-silico*molecular docking analysis of the representative polyphenols and compounds isolated from *G. indica*fruits with pancreatic lipase.

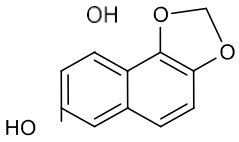
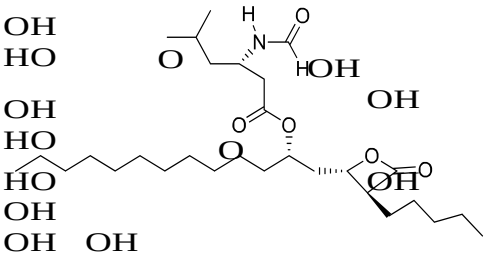
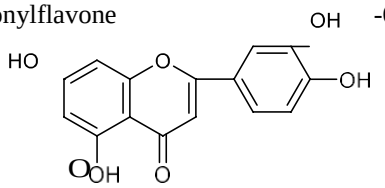
Sample Name and Structure	Dockingscore	Hydrogenbond interactions	π –π stacking	Bindingenergy (Kcal/mol)
Cyanidin 	-8.222	ASP 79 GLY 76 PRO180 SER152	PHE 215 HIE151 HIS 263	-38.0386
Proanthocyanidin 	-6.545	ASP79 PHE77 LEU153 GLU179 TYR114 SER152	-	-49.9582
Flavanonylflavone 	-6.529	ASP 79 GLY76 PHE77 HIS 263 ALA 259	HIS 263	-36.4929

Table 6.1 (continued): *In-silico*molecular docking analysis of the representative polyphenols and compounds isolated from *G. indica*fruits with pancreatic lipase.

Sample Name and Structure	Dockingscore	Hydrogen bond interactions	π –π stacking	Bindingenergy (Kcal/mol)
Orlistat	-5.402	Asp 79, Leu153 Phe77		-47.4155
Luteolin (1)	-9.46	HIE151 GLY76	HIE 151 PHE 77 HIP 263	-44.021
Napthyldioxolol (2)	-8.19	SER 152	-	-38.500
Oleantrienoic acid glucoside (3)	-10.11	PHE 77 HIE 151	-	-35.000

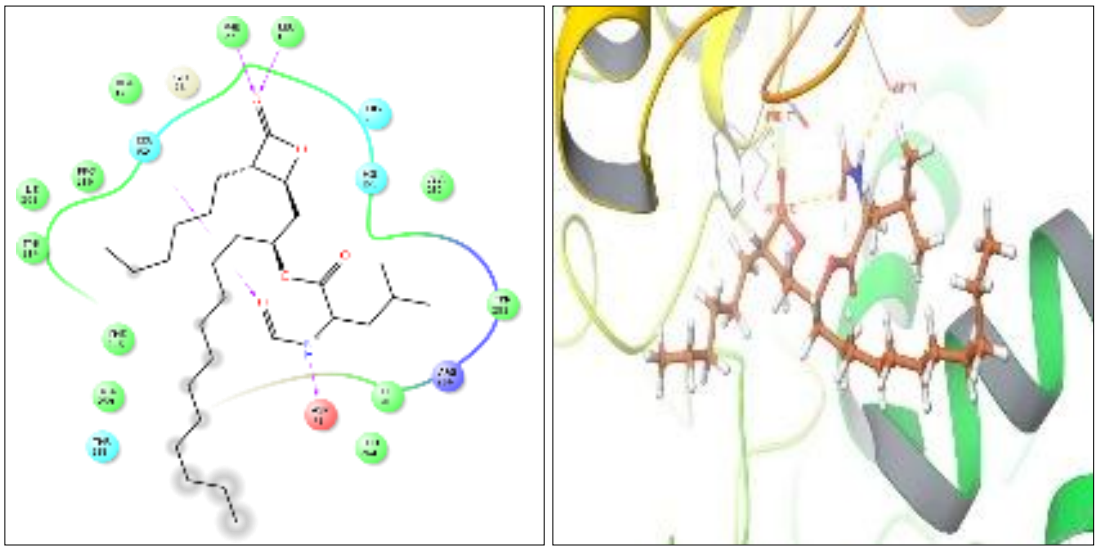


Figure 6.1: Docking studies of orlistat (A), representative polyphenols (B-D) and compounds isolated (E-G) from *G. indica*fruits with pancreatic lipase

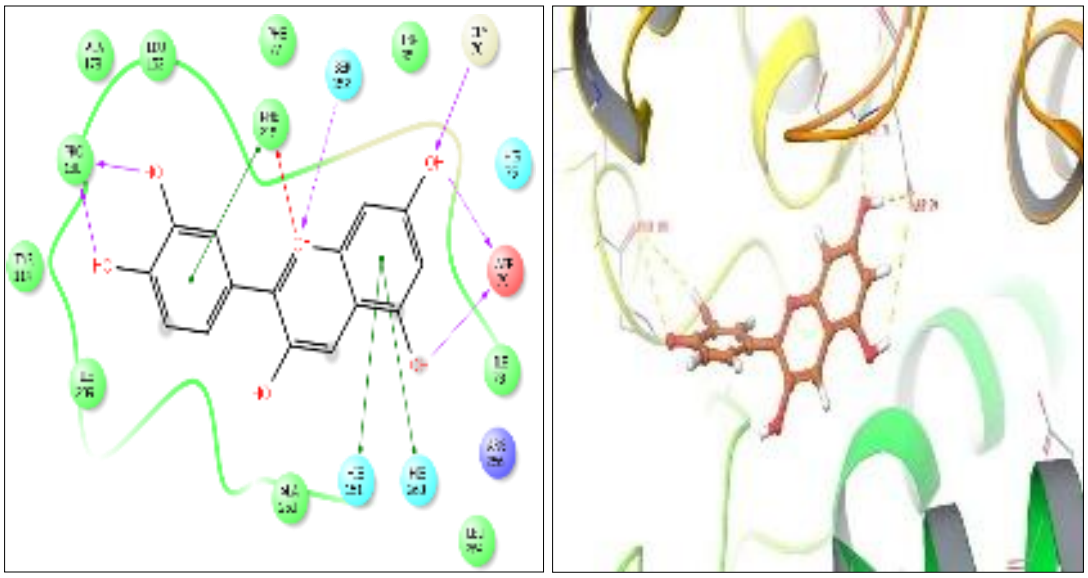


Figure 6.1.A: Schematic diagram 2D (left) and 3D (right) of orlistat inside the pancreatic lipase receptor pocket (PDB ID - 1LPB). Orlistat is showing key hydrogen bond interaction with Asp 79, Leu153, Phe77 and Ser 152 (purple colour dash line).

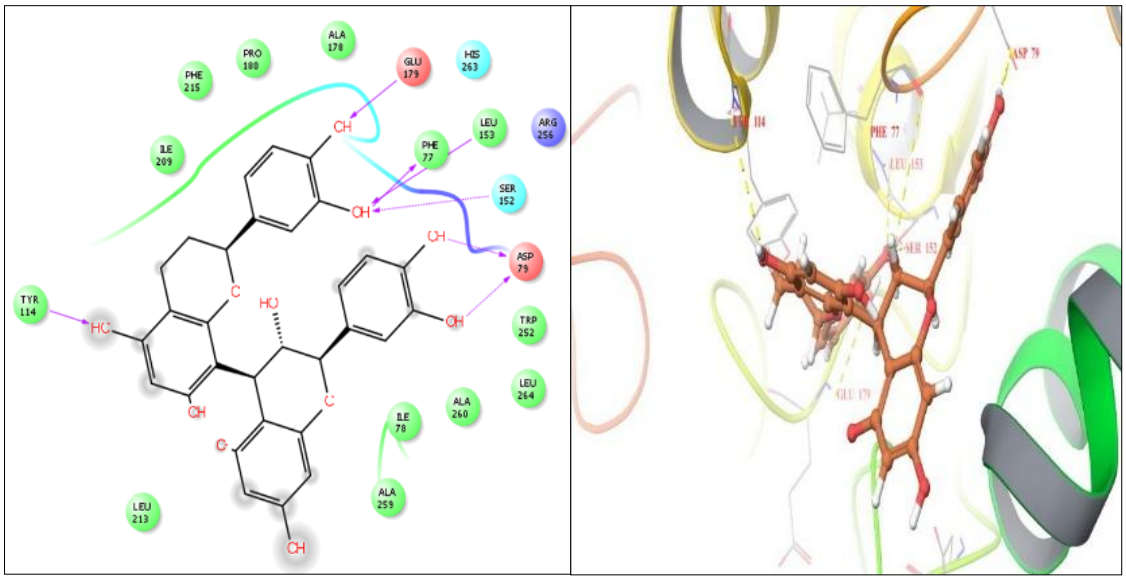


Figure 6.1.B: Schematic diagram 2D (left) and 3D (right) of cyanidin inside the pancreatic lipase receptor pocket (PDB ID - 1LPB). Cyanidin is showing key hydrogen bond interaction with Asp 79, Gly76, Pro180 and Ser152 (purple colour dash line) and π- π stacking (green colour solid line) with Phe 215, Hie151 and His 263.

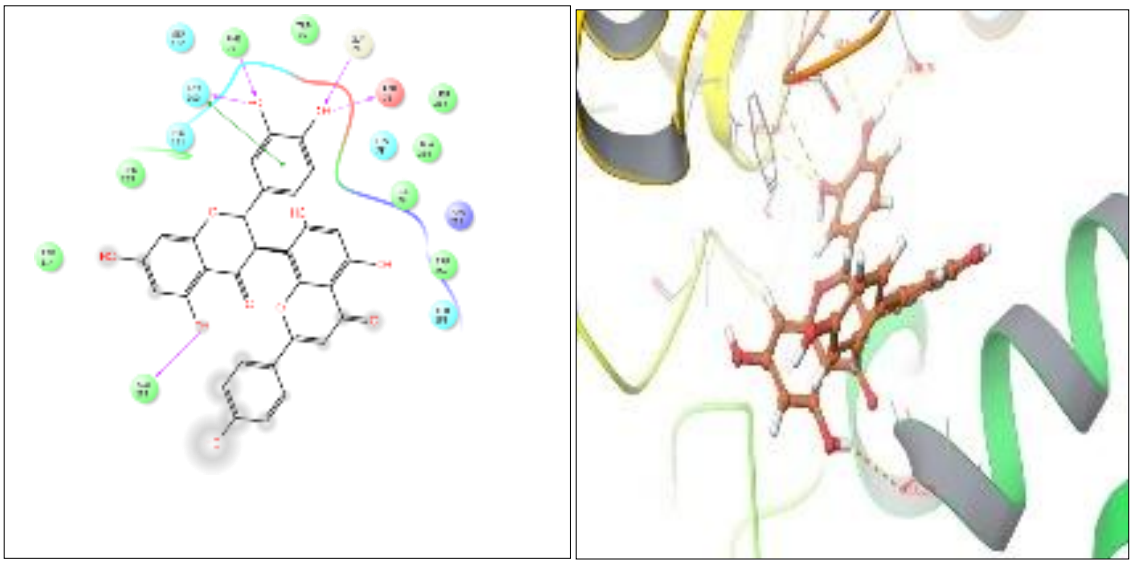


Figure 6.1.C: Schematic diagram 2D (left) and 3D (right) of proanthocyanidin inside the pancreatic lipase receptor pocket (PDB ID - 1LPB). Proanthocyanidin is showing key hydrogen bond interaction with Asp 79, Phe77, Leu 153, Glu179, Tyr114 and Ser152 (purple colour dash line).

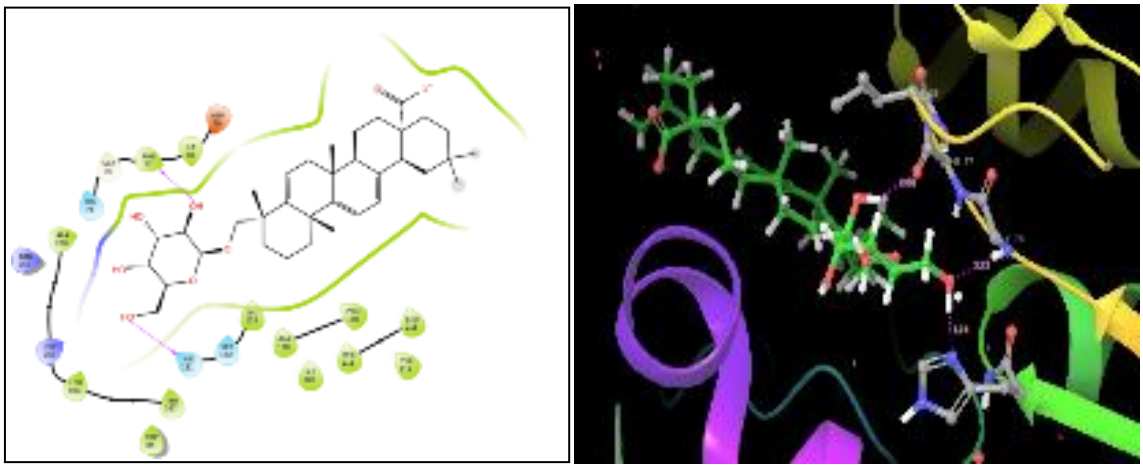
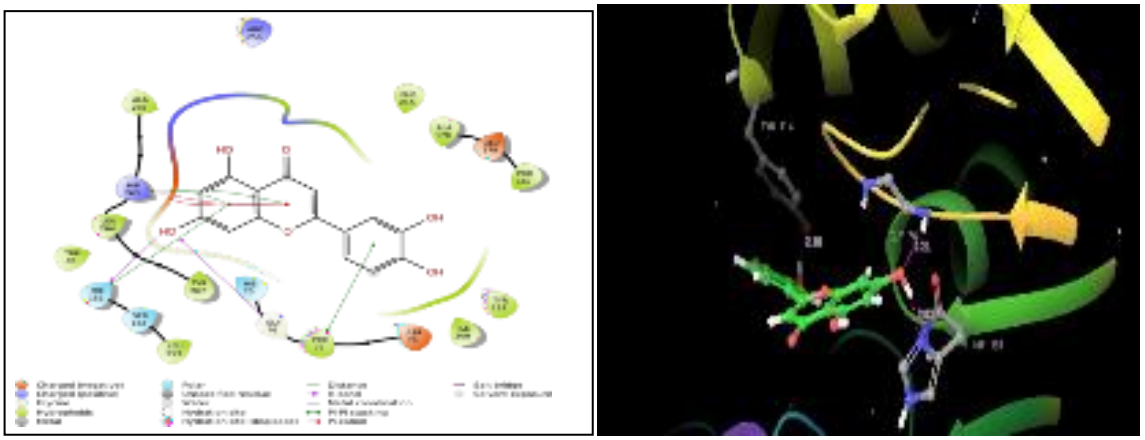


Figure 6.1.D: Schematic diagram 2D (left) and 3D (right) of the drug flavonoyl flavone inside the pancreatic lipase receptor pocket (PDB ID - 1LPB). Flavonoyl flavone is showing key hydrogen bond interaction with Asp 79, Gly76, Phe 77, His 263, Ala 259 (purple colour dash line) and π-π stacking with His 263(green colour solid line).

Figure 6.1.E: Schematic diagram 2D (left) and 3D (right) of oleantrienoic acid glucoside inside the pancreatic lipase receptor pocket (PDB ID - 1LPB).

Figure 6.1.F: Schematic diagram 2D (left) and 3D (right) of luteolin inside the pancreatic lipase receptor pocket (PDB ID - 1LPB).



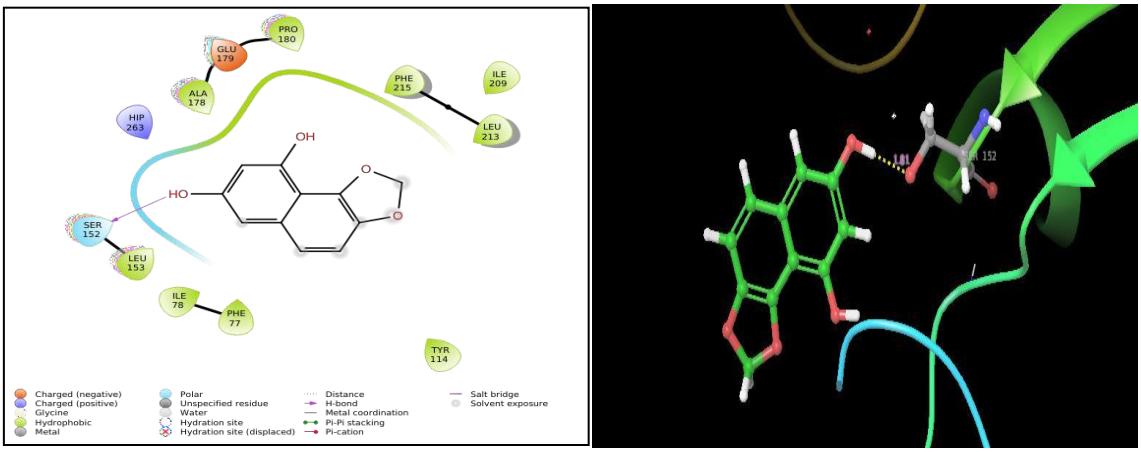


Figure 6.1.G: Schematic diagram 2D (left) and 3D (right) of Napthyldioxolol inside the pancreatic lipase receptor pocket (PDB ID -1LPB).

IN VITRO ANTI-OBESITY ACTIVITY

The investigational research for anti-obesity medication is often aimed at the modulation of energy homeostasis by stimulating catabolic or inhibiting anabolic pathways. Calories intake mainly include fatty components (triglycerides) and carbohydrates. Two gastrointestinal enzymes namely pancreatic lipase (PL) and α- amylase are involved in metabolism of fats and carbohydrates. PL hydrolyses the triglycerides whereas α-amylase enzyme is accountable for the digestion of carbohydrates in human body. The therapeutic approach to control obesity lies in the inhibition of these enzymes, so as to reduce the energy intake without altering central mechanisms (Marrelli et al., 2013).

The purpose of this study was to assess the anti-obesity effect of polyphenol rich fraction of *Alpiniagalanga*rhizomes, *Garcinia indica*fruits and *Glycyrrhizaglabra*roots by determining their effect on digestive enzymes (PL and amylase).

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), FBS (Fetal Bovine Serum), Insulin, Oil Red O stain, MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide), lysis buffer, pancreatic α-amylase, pancreatic lipase, acarbose, DNS color reagent, orlistat, acarbose, p-NPB (p - Nitro phenyl butyrate), nonidet P-40, isopropyl alcohol, sodium nitroprusside, sodium hydroxide, *n*-naphthyl ethylene diamine dihydrochloride, Dithiothreitol (DTT), ascorbic acid, quercetin, diclofenac sodium, gallic acid etc. were purchased from Sigma. EDTA, glucose & antibiotics from Hi- Media Laboratories Ltd., Mumbai, India and dimethyl sulfoxide (DMSO) & propanol procured from E. Merck Ltd., Mumbai, India. Other chemicals used in this study were of analytical grade. *In-vitro* antioxidant activity was determined using Shimadzu UV- Vis spectrophotometer and MTT assay was carried out in Beckmann coulter Elisa plate reader (BioTek Power wave XS).

Fractionation and isolation of compounds

Dried *A. galanga*rhizomes, *G. indica*fruits and *G. glabra*roots (1 Kg) were dried, pulverized and extracted with methanol (10 l) till exhaustion using Soxhlet apparatus. The methanolic extract (AGME, GIME and GGME respectively) were filtered andconcentrated *in vacuum* using Rotavapor (Buchi, Switzerland) to get a semisolid residue. The *residue* was then dissolved in distilled water and partitioned sequentially with different solvents to yield ethyl acetate, chloroform, butanol and aqueous fractions that were designated as (AGEF, AGCF, AGBF, AGAF), (GIEF, GICF, GIBF, GIAF) and (GGEF, GGCF, GGBF, GGAF) respectively. The fractions were concentrated using Rotavapor and the air dried residues were kept in a refrigerator till further use.

Estimation of Total phenolic content (TPC)

TPC was estimated in all the fractions using Folin-Ciocalteu method (Singleton et al., 1999). Each fraction was diluted to prepare a stock solution (10 mg/ml). Test sample solution (100 µl) of different fractions was mixed with 500 µl of Folin-Ciocalteu's reagent and 400 µl sodium carbonate (20%) and incubated at 25-27 ºC for 90 min. The absorbance was measured at 760 nm using UV-Vis spectrophotometer (JASCO V-550, Japan). TPC was expressed as milligram gallic acid equivalent (GAE) per gram of testsamples.

Estimation of Total flavonoid content (TF)

The aluminiumtrichloride assay was performed to measure TFC of all the fractions with slight modifications (Zhishen et al., 1999). Test samples was dissolved in 10% DMSO to yield 500 µg/ml solution that was mixed with 150 µl of 5 M $NaNO_2$. After 5 minutes, 150 µl of 10 % aqueous $AlCl_3$ was added to the mixture followed by 1 ml of 1 M NaOH. After 15 min of incubation, the absorbance was measured at 510 nm on UV-Vis spectrophotometer. All measurements were repeated thrice. A calibration curve of standard reference was assessed as quercetin equivalent in milligrams per g of testsample.

ENZYME INHIBITION ASSAY

In vitro pancreatic lipase inhibitory assay

PL inhibitory activity of all samples were determined by spectroscopic estimation (Winkler and Stuckmann, 1979). Briefly, p-nitrophenolpalmitate (pNPP) hydrolysed by PL to p-nitrophenol (pNP) is monitored at 410 nm. The assay mixtures composed of 1.8 ml sodium phosphate buffer (0.05 M, pH 7.6), 1.15 mg/ml sodium cholate, 0.55 mg/ml arabic gum, 0.2 ml pNPP in isopropanol (0.01 M) and 0.02 ml of samples (atdifferent concentration) were incubated at 37 □ C. Then, 0.02 ml of the PL solution in sodium phosphate buffer (50 mg/ml) was added to initiate the reaction. After 5 min of incubation at 37 □ C, the absorbance was recorded at 410 nm. The control reaction was carried out without adding samples.The percentage of enzyme inhibition was determined as:

Where A_{test} and $A_{control}$ represent the absorbance of reaction mixture with and without test sample, respectively. IC_{50} values represented concentration of samples required for inhibiting 50% of enzyme activity under the assayconditions.

In vitro α-amylase inhibitory assay

α-amylase inhibition assay was carried out as per the method described by Dong et al., 2007. α-amylase (40 µl of 5U/ml) in 0.36 ml of sodium phosphate buffer (0.02 M, pH 6.9 containing 0.006 M NaCl) was mixed with 0.2 ml of samples or acarbose. The mixture was incubated for 20 min at 25 □ C and 300 µl of starch solution (1% in 0.02 M sodium phosphate buffer) was added, the mixture was incubated again for 20 min at 25 □ C.The reaction was stopped by addition of 0.2 ml of dinitrosalicylic acid (DNS) and the contents were kept in a boiling water bath for 5 min and the absorbance was recorded at 540 nm The control reaction was carried out without addingsamples.

The percentage of enzyme inhibition was determined as:

Where A_{test} and $A_{control}$ represent the absorbance of reaction mixture with and without test sample, respectively. IC_{50} values represented concentration of samples required for inhibiting 50% of enzyme activity under the assayconditions.

Cell viability MTT assay

3T3-L1 pre-adipocyte cells were obtained from the National Centre for Cell Science (NCCS) Pune. Cells were maintained in DMEM containing 10% foetal bovine serum and 1% penicillin and streptomycin in a humidified 5% CO_2 atmosphere at 37 □ C. The cells were cultured at in a 96-well plate (5 x 104 cells/ml) to evaluate the cytotoxicity. of samples. The cells were treated with different concentrations ranging from 100 to 2000 mg/ml for 48 h. It was followed by the incubation of cells with MTT solution for 3 h at 37 □ C under a humidified 5% CO_2 atmosphere. The supernatants were aspirated, DMSO was added to each well and the absorbance was measured at 570 nm using a micro-well plate reader. The cytotoxicity of test samples was estimated by comparing their absorbance values with the untreated control cells (Ganjayi et al., 2017).

***In vitro* anti - inflammatory studies of AGEF**

AGEF was also screened for anti-inflammatory activity utilizing hindrance of albumin denaturation strategy. The response blend comprises of 1 mL of 1% aqueous arrangement of bovine serum albumin division, 1 mL of PBS at pH 6.4 and 1 mL of different concentration of AGEF was used (Figure 8) utilizing DMSO and dis-tilled water individually. 10 g/ml diclofenac sodium was used as the standard drug and distilled water as the control. Then the samples were incubated at (37 ± 2) ° C for 15 min and then heated at 70 °C for 5 min. After bring down the temperature of the AGEF, the absorbance was measured at 660 nm. The experiment was performed in triplicate. Percent inhibition of AGEF was calculated (Hiba et al.,2014).

Adipocyte differentiation

For adipocytes differentiation, cells were grown in DMEM with 10% FBS until confluence was reached. Two days after confluence, pre-adipocytes were stimulated to differentiate in the presence of DMEM, 10% FBS, 0.5 mM 3-isobutyl-1-methyl-xanthine, 10 µg/mL insulin and 1 mM dexamethasone for 2 days. Cells were then maintained in DMEM,10% FBS and 10mg/mL 1 M insulin for another 2 days, followed by culturing with DMEM, 10% FBS for an additional 4 days. All the media used contained 100 IU/mL penicillin and 100 µg/mL streptomycin. AGEF was dissolved in DMSO final concentration: 0.1% in media). The cultures were treated with test samples for the entire culture period (day 0-8). The size and quantity of the stained lipid droplets were observed under a bright field microscope.

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