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Antilipase, antiproliferatic and antiradical activities of methanolic extracts of *Vinca major*

Sukhdev Singh, Jyoti Gupta and Shamsher S. Kanwar

Abstract
Plants offer us bioactive molecules those may serve as safer therapeutics to combat existing new world diseases, and obesity is the major concern among them. Pancreatic lipase inhibitors from plant sources may prove as promising side effects lacking antiobesity therapeutics. Very rare knowledge is elaborated about ancient plants which are used traditionally as ailments to treat various problems by tribal communities in Himalayas region. Keeping this in mind, present study was conceived with the objective of antilipase screening of ethnobotanic flora of Western Himalaya (Shimla region). Antilipolytic function of *Vinca major* was assessed using porcine pancreatic lipase (PPL; triacylglycerol lipase, EC 3.1.1.3) in an *in vitro* assay system with *p*-nitrophenyl palmitate (*p*-NPP) as a substrate. Amongst screened plants, *Vinca major* flower extract (VFE) exhibited the highest inhibitory effect in a reversible manner with an IC_{50} value of 403 µg/ml. Kinetic studies demonstrated that VFE exerted a mixed type of inhibition on PPL. Additionally, antiproliferative, antioxidant and hemolytic effects of VFE were also assessed *in vitro*. VFE reduce growth of Hep-2 cells with IC_{50} value of 228 µg/ml. Antioxidant activities were evaluated by using different free radicals (DPPH, H_2O_2, superoxide and hydroxyl). The antioxidant activity of *Vinca* leaf extract (VLE) was proved to be better than the flower extract but it appeared to be lower when compared to ascorbic acid. VLE eliminated DPPH, H_2O_2, superoxide and hydroxyl radicals in dose dependent manner with IC_{50} values of 170, 600, 421 and 611 µg/ml, respectively. Phytochemical constituents were quantified in *Vinca* flower and leaf extracts. Quantifications of total phenolics, tannins, flavonoids, alkaloids and saponins were done by taking tannic acid, quercetin, atropine and diosgenin as reference molecules. Both VLE and VFE possessed alkaloids and flavonoids in abundance respectively. VFE possessed mild hemolytic activity with an IC_{50} value of 2.40 mg/ml when assessed *in vitro*.

Keywords: Antilipase, PPL, *Vinca major*, antiproliferative, antioxidant, alkaloids, flavonoids, hemolytic.

1. Introduction
Obesity, the New World Syndrome, is now being recognized as a major health concern in both developed and developing countries [1]. IASO/IOTF analysis predicted that approximately 1.0 billion adults are currently overweight and amongst these 475 million are obese [2]. In the European Union, approximately 60% of adults and over 20% of school-age children are overweight or obese. In England over a quarter of adults (26%) were reported to be obese in 2010 [3]. In 2009-2010, 35.7% of U.S. adults and almost 17% of youth were recorded as obese [4]. Indians are also reported for obesity and its associated consequences [5]. Also, there is a constant rise in obesity related deaths each year on account of coronary artery disease, stroke, type-2 diabetes, heart failure, dyslipidemia, hypertension, reproductive & gastrointestinal cancers, gallstones, fatty liver disease, osteoarthritis and sleep apnea [6]. It has been found that obesity also promotes prostate cancer [7]. Retardation of nutrient absorption and digestion may be used as an approach to manage obesity and related disease. Medication is also available as therapeutic compounds that can block/ inhibit fat digestion [8]. Orlistat, which is a saturated derivative of Lipstatin [9] is currently the only approved drug that lowers the body weight when taken under suitable dietary advice [10, 11]. This medication, although blocks the absorption of dietary fat yet it causes unpleasant side effects (greasy stool) and it also needs the supplementation of diet with fat-soluble vitamins. Its use is associated with high rates of side-effects on gastrointestinal tract and on the kidneys.
There are some issues, which need to be kept in mind before any effective new therapy will be widely accepted. Firstly, it should not be addictive. The second issue concerns the plateau of body weight that is reached when homeostatic mechanisms in the body come into play and

stop further weight loss and the final issue is the toxicity associated with many anti-obesity drugs. Because of the latter, plants may prove to as alternative source(s) of a variety of antilipolytic therapeutic molecule(s) that can either inhibit the pancreatic lipase and/ or the uptake/ absorption of fat(s) or both. Natural compounds from plants and microbes can be developed as clinical products [12]. Phytochemicals present in plants offer us the safer natural products. These bioactive compounds can be extracted and purified in many ways [13]. For the obesity pandemic these bioactive compounds may be targeted on lipid metabolic pathways. A very large pool of enzymes related to fat digestion has been discovered and can be targeted for development of therapeutics for obesity and related diseases [14]. Pancreatic lipase, a key mammalian enzyme, which is responsible for hydrolysis of majority of dietary fats [15], may be targeted for the obesity pandemic. Like, Orlistat, Cetilistat also acts by inhibiting gastrointestinal (GI) and pancreatic lipases but have serious side effects including oily stools, diarrhea, abdominal pain, fecal spotting and some hepato-toxicity [16, 17].

So in continuing search for newer pancreatic lipase inhibitors, we screened western Himalayan flora of Shimla region for antilipase activity. Among tested plants, 3 species (*Arisaema dracontium*, *Urtica dioica* and *Vinca major*) showed promising antilipase potential. Amongst these, flower extract of *Vinca major* exhibited highest antilipolytic activity. So the objectives of our research work were to investigate the antilipolytic potential of VFE on PPL and other commercial lipases (Steapsin, Lipolase*100L) and quantification of major phytochemical components. Additionally, anti-proliferative, antioxidant and hemolytic effects of VFE were also assessed *in vitro*.

2. Materials and methods

2.1 Study site and plant collection

The study was conducted in rural villages of district Shimla (India) located at 31.61 ^{0}N 77.10 ^{0}E. The altitudinal range of these areas lies between 1000 to 2500 m above mean sea level. The climate of the selected areas is cool and dry. The region receives moderate rainfall during monsoon of which the bulk is received during the months of July- August. Snowfall is received during the months of December-February. Ethnomedicinal values and local names of collecting plants were addressed from senior citizens aged above 60 yrs. Scientific names of plants were addressed from available databases on Western Himalayan flora [18].

2.2 Preparation of methanol extracts and screening of plants for antilipase activity

Collected plants were shade dried and grounded (whole plant material) to fine powder. Each powdered material was extracted two times with methanol and extracts were obtained through the removal of the solvent by evaporation at room temperature. The percentage yields of plant extracts were in the range 0.5 to 0.8% (w/w). PPL inhibition by each plant extract was assayed at 250 µg/ml concentration. Before bioassays, the extracts were prepared as a stock solution at a concentration of 0.1 mg/ml in phosphate buffer (pH 7.2).

2.3 Chemicals

Porcine pancreatic lipase, Steapsin and Dinitrophenyl hydrazine (SRL, Mumbai, India); Lipolase*100L (Novozymes, Bangalore, India); O'Stat (Orlistat; ARISTO Pharmaceuticals, Mumbai, India); *p*-NPP (Lancaster Synthesis, England); MTT, Quercetin and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich Co., USA); Trypsin, Tannic acid, Nitro blue tetrazolium, Diosgenin and Vanillin (Himedia, Mumbai, India); DMSO and hydrogen peroxide (Fisher Scientific, Mumbai, India) were procured from various commercial suppliers. All other chemicals were of analytical grade and were used as received.

2.4 *In vitro* assay for measurement of pancreatic lipase inhibitory activity

The initial and residual PPL activity was measured using *p*-NPP as a substrate by a previously described method [19]. PPL stock solution (5 mg/ml) was prepared in 1 mM potassium phosphate buffer (pH 7.2) and stored at -20 ^{0}C. To determine the lipase inhibitory activity, plant extract (50, 100, 150, 200, 250, 300 µg/ml) or Orlistat (50, 100, 150, 200, 250 and 300 µg/ml) as a positive control, were pre-incubated with PPL (20 µl) for 30 min in a potassium phosphate buffer (1 mM, pH 7.2) at 37 ^{0}C before assaying the PPL activity. The reaction was then started by adding *p*-NPP (40 µl; 10 mM) as substrate in a final volume of 3.0 ml. After incubation at 37 ^{0}C for 5 min, the amount of *p*-nitrophenol released in the reaction mixture was measured at 405 nm using a UV-Visible spectrophotometer (3000+, Lab India, Mumbai). Each experiment was performed in duplicates and mean values were presented. The percent PPL inhibition was calculated according to the following formula:

$$\text{Percent inhibition (I \%)} = 100 - [(B/A) \times 100]$$

Where A is the activity without plant-extract or Orlistat; B is the activity with plant extract or Orlistat. Control contained all above stated reaction components, except the enzyme.

2.5 Antilipolytic effects of *Vinca major*/ O'Stat on Steapsin and Lipolase

Inhibitory effects of *Vinca major* tissue extracts and Orlistat were examined on other lipolytic enzymes (Steapsin and Lipolase*100L) also. Activities of these lipases were measured as above using *p*-NPP.

2.6 Kinetic analysis of lipase-inhibiting activity of VFE

In order to measure the inhibition mode by methanolic extract of *Vinca* flowers, pancreatic lipase activity was assayed with increasing concentrations of *p*-NPP as substrate (2.5, 5, 10 and 20 mM) in the absence and presence of three different concentrations of the extracts (0.20, 0.25 and 0.30 mg ml^{-1}). Inhibition mode was determined by double-reciprocal Lineweaver-Burk plot analysis of the data resulted from enzyme assays containing various concentrations of *p*-NPP and extracts according to the Michaelis-Menten kinetics. Above assays were performed in triplicates and mean values were presented.

2.7 Cytotoxicity of VFE on Hep-2 cell line

Established Hep-2 cell line was used to evaluate the antiproliferative activity of VFE. Hep-2 cells were grown in DMEM (Himedia) supplemented with 10% heat inactivated

foetal bovine serum (PAA Laboratories), incubated in a humidified atmosphere of 4% CO_2 at 37 ^{0}C. The cytotoxicity of VFE was measured using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The assay detects the reduction of MTT by mitochondrial dehydrogenase to a blue formazan product which reflects the normal function of mitochondria and cell viability. For testing, cells were washed in EMEM, harvested by trypsinization, plated in 96 well microplate. After 48 h incubation when monolayer was observed cells were treated with different concentrations (10, 30, 50, 70, 90, 110, 130 and 150 µg/ml) of flower extract and reincubated for 24 h. Then 20 µl of MTT solution (5 mg/ml) were added to each well. After 1 h incubation with MTT, medium were aspirated from each well and 100 µl DMSO was added to each well and gently shaken. Reduced MTT was assayed at 570 nm using a microplate reader (Thermo Electron Corporation). Culture medium containing methanol was used as solvent control and untreated cells preparation was used as negative control. % Cells inhibition was calculated according to following formula:

% Cells inhibition= [A_{570} of control- A_{570} of sample/ A_{570} of control] × 100

2.8 Preliminary phytochemical tests

Qualitative phytochemical analysis was performed according to standard procedures available to identify bioactive constituents of *Vinca major* [20]. $FeCl_3$ test was done for phenols and tannins; ammonia and Na OH test for flavonoids; foam test for saponins; Wagner's reagent test for alkaloids; Salkowski and Liebermann-Burchard test for terpenoids and steroids; Keller-Killani's test for glycosides; Ninhydrin test for proteins; Fehling's, Molisch's and iodine tests to detect carbohydrates and di-nitrophenyl hydrazine test to detect carbonyls.

2.9 Quantitative phytochemical analysis

2.9.1 Estimation of total phenolic content and tannins

Total phenolic content was determined by using the Folin-Ciocalteu reagent [21]. 100 mg *Vinca* tissue extract was taken in a glass beaker of approximately 50 ml capacity. Ten ml of aqueous acetone (70%) was added and the beaker was kept on magnetic stirrer for 45 min. The contents of the beaker were subjected to centrifugation for 10 min at 1500 g at 4 ^{0}C. Supernatant was collected and tubes were kept on ice. Phenolic and tannin contents were quantified in 100 µl aliquots of this supernatant. Suitable aliquots of tannic acid (20, 40, 60, 80 and 100 µg) were made up to 0.5 ml volume with distilled water and 0.25 ml Folin-Ciocalteu (1 N) reagent was added. Then 1.25 ml of sodium carbonate (20%) solution was added to the reaction mixture, vortexed properly and A_{725} values after 40 min were recorded. Amount of total phenols in tissue extracts was calculated as tannic acid equivalent from standard calibration curve.

For tannin quantification, 100 mg Poly vinyl pyrrolidone (PVP) was added to 1 ml tannin containing extract. Reaction mixture was properly vortexed, kept on ice (for 20 min) and centrifuged (1500 g, 10 min). Amount of phenolics other than tannins were measured at A_{725} with the help of tannic acid standard curve as stated above.

2.9.2 Estimation of total flavonoid content

Total flavonoid content was determined using $AlCl_3$ [22] with slight modifications by using Quercetin as a standard. To 100 mg tissue extract, 5 ml ethyl ether was added and the content was stirred for 1 h on a magnetic stirrer. The suspension was centrifuged (3000 g, 20 min), supernatant was discarded and ether was evaporated overnight. Thereafter, 5 ml of methanol was added to the extract and the content was filtered through Whatman filter paper no. 1. This filtrate was diluted up to 50 ml with distilled water and 2.5 ml aliquot was taken for flavonoid quantification. $NaNO_2$ (5%; 150 µl) was added to plant extract (2.5 ml) and incubated for 5 min at room temperature. Later, 150 µl $AlCl_3$ (10%) was added and after 5 min, the reaction mixture was treated with 1 ml (1 M) Na OH. Finally, the reaction mixture was diluted to 5 ml with distilled water and A_{510} was recorded. The flavonoid content was calculated from a reference profile of Quercetin (8- 80 µg).

2.9.3 Estimation of alkaloids

Alkaloid contents were assayed by a previously reported method [23] with slight modifications and Atropine was used as a standard. Tissue extract stock solution (0.1 mg/ml) was prepared in phosphate buffer (pH 7.2). One ml of this solution (or atropine solution) was mixed with 0.5 ml Bromocresol green (BCG, 0.1 mM). Reaction mixture was shaken and the complex formed was extracted with 1.5 ml chloroform. The absorbance of the complex in chloroform was measured at A_{470}. Experiments were performed in triplicates and the results were averaged.

2.9.4 Saponin quantification

Total saponins were extracted and quantified (using Diosgenin) in *Vinca major* tissue extracts by a previously reported method [24] with minor modifications. In brief, tissue samples were defatted two times with *n*-hexane in a ratio of 1:5. Then dried defatted sample was extracted two times with aqueous methanol (50%, 20 ml) and stirred overnight on a magnetic stirrer at room temperature. Suspensions were then centrifuged (3000 g, 10 min, 4 ^{0}C) and supernatant was collected. Methanol was evaporated from this supernatant and the aqueous phase was centrifuged to remove insoluble materials. An equal volume of chloroform was added to this aqueous layer in a separating funnel to remove pigment. Finally, these concentrated saponins were extracted two times with *n*-butanol and dried fractions were obtained by evaporating the *n*-butanol. These dried fractions were preserved at -20 ^{0}C and 1 mg/ml stock solution was prepared in aqueous methanol at the time of quantification. To 1 ml aliquot of this stock solution, vanillin reagent (8%, 0.25 ml) & sulphuric acid (25%, 0.75 ml) was added, and reaction mixture was incubated at 60 ^{0}C in a water bath. After 10 min incubation, tubes were cooled and absorbance in each case was read at 544 nm. A standard curve was prepared by assaying different concentrations (50, 62.5, 75, 87.5, 100, 112.5 and 125 µg) of Diosgenin.

2.10 Antioxidant assays

2.10.1 DPPH radical scavenging activity

DPPH radical scavenging activity was assessed in *Vinca* tissue extracts by a previously reported method [25]. Tissue extracts at various concentrations (50 to 400 µg/ml) were

mixed in 1 ml of freshly prepared 0.5 mM DPPH ethanolic solution and 2 ml of 0.05 M Tris buffer, pH 7.5. The resulting solutions were incubated in dark at 37 ^{0}C for 20 min and A_{517} values were recorded. Ascorbic acid was used as a positive control. Lower A_{517} values represented higher DPPH scavenging activity. The % DPPH radical scavenging activity of plant extract was calculated from the decrease in absorbance in comparison to the control using the following equation:

DPPH scavenging effect (%) = $[A_0 – A_1 / A_0] \times 100$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of plant extract.

2.10.2 Hydrogen peroxide (H_2O_2) radical scavenging activity

H_2O_2 radical scavenging activity was also determined [26]. Selected concentrations (50 to 400 µg/ml) of tissue extracts or ascorbic acid in methanol was added to 2 ml of H_2O_2 (10 mM) in phosphate buffer saline (pH 7.2). After 10 min the A_{230} was recorded and scavenging activity was calculated as follows:

H_2O_2 radical scavenging effect (%) = $[A_0 – A_1 / A_0] \times 100$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of plant extract.

2.10.3 Iron reducing power assay

Iron reducing potential of *Vinca* tissue extracts was determined as reported previously [27]. Tissue extract or ascorbic acid (50 to 400 µg/ml) was mixed with 1 ml of phosphate buffer (1 mM, pH 6.6) and 1 ml of 1% (w/v) K_3 Fe (CN) $_6$. This reaction mixture was incubated at 50 ^{0}C for 20 min. After incubation period, 1 ml of 10% TCA was added and the reaction mixture was centrifuged at 1500 g for 10 min. Now the upper layer was mixed with 1 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1% w/v) and thoroughly mixed. The A_{700} was recorded and iron reduction was calculated using following equation:

Iron reduction (%) = $[A_S – A_C / A_C] \times 100$

A_S was the absorbance of the sample and A_C was the absorbance of the control.

2.10.4 Superoxide radical scavenging activity

This assay was performed by a previously reported method [28]. Superoxide radical is generated when sodium hydroxide was added to air saturated DMSO. The generated superoxide remains stable in solution, which can reduce NBT into formazan dye at room temperature and this formazan can be measured at 560 nm. Briefly, to the reaction mixture containing 1 ml of alkaline DMSO (1 ml DMSO in 5 mM NaOH) and plant extract or ascorbic acid (50 to 400 µg/ml), 0.1 ml of NBT (1 mg/ml) was added. The scavenging activity was calculated using following equation:

Superoxide radical scavenging effect (%) = $[A_0 – A_1 / A_0] \times 100$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample.

2.10.5 Hemolytic activity of flower extract

In vitro hemolytic activity was performed by a spectrophotometric analysis [29]. Three milliliters of blood ('O' Rh +ive) were collected from a healthy individual. The blood was centrifuged at 3000 g for 5 min. The pellet was washed three times with sterile phosphate buffered saline solution (pH 7.2) by centrifugation at 3000 g for 3 min. The cells were resuspended in PBS to 1%. A volume of 0.5 ml of the cell suspension was mixed with 0.5 ml of the varying concentrations of flower extracts (50, 100, 200, 300 400, 500, 600 and 700 µg/ml). The suspensions were incubated for 30 min at 37 °C followed by centrifugation at 3000 g for 5 min. The free hemoglobin in the supernatant was measured at A_{540}. The reaction system comprising phosphate buffered saline and distilled water was used as a negative control. Each experiment was performed in duplicate and mean values were presented. The level of percent hemolysis (% Hemolysis) by the extract was calculated according to the following formula:

% Hemolysis = $[A_T – A_S / A_W – A_S] \times 100$

Here: A_T is the absorbance of test sample (flower extract)
A_S is absorbance of the control (saline control)
A_W is the absorbance of the control (water control).

3. Results and Discussion

3.1 Antilipolytic activity of *Vinca major*

Obesity mainly results from the disequilibrium between energy intake and its expenditure. It is believed to be associated with numerous diseases, including hyperlipidemia, hyper-cholesterolemia, type 2 diabetes and prostate cancer [7]. 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 excess calories. Phytochemicals provide an exciting opportunity to develop safer therapeutics [12]. As part of the continuing search for discovery of biologically active anti-obesity agents from natural herbal resources, crude extracts in methanol were prepared for a few selected ethnobotanic plant species found in Shimla and their antilipase activities were investigated at 250 µg/ml concentration (Table 1). Among screened plants, three species (*Arisaema dracontium*, *Urtica dioica* and *Vinca major*) markedly inhibited PPL. Amongst these, *Vinca major* exhibited promising antilipase activities. PPL inhibition by *Vinca major* tissue extracts was recorded (Figure 1) and IC_{50} values (the concentration required to inhibit the original lipase activity by 50%) were also determined (Table 2). Antilipolytic effects of VFE were tested against other commercial lipases also (Figure 2 & 3). Moreover, the antilipolytic activity of VFE appeared to be a reversible one (data not shown) with an IC_{50} value of 403 µg/ml. Both VLE as well as VFE were comparatively less effective than Orlistat (IC_{50} = 9.45 µg/ml) in their antilipolytic function (Table 2). Orlistat is a hydrogenated derivative of Lipstatin and the only lipase inhibitor currently approved for a long-term treatment of obesity. Unfortunately, Orlistat has many serious side effects, such as gas formation/ flatulence with oily spotting, stomach

people under obesity treatment [8]. VFE was further investigated for mode of lipase inhibition, its anti-proliferate & antioxidant potential and major phytochemical constituents.

Table 1: Ethnomedicinal values and lipase inhibitory (LI) effects of Shimla region plants.

S. No.	Botanic name/ local name[#]	Family	Uses[†]	LI* (%)
1.	*Abies pindrow*/ Fir	Pinaceae	In cough, asthma and other bronchitis problems	0.5
2.	*Adiantum capillus-veneris*/ Hanspari	Adiantaceae	In treating burns, dysentery, throat infections	-9.4
3.	*Arisaema dracontium* / Chitkabri naag	Araceae		19.3
4.	*Berberis aristata*/ Kasmal	Berberidaceae	hypoglycaemic, lowering of blood pressure, skin diseases, in eye problems, in jaundice	10.7
5.	*Bidens pilosa*/ Badgumbri	Asteraceae		4.5
6.	*Boenninghausenia albiflora*/ Pissumar	Rutaceae	wound healing, antiseptic, juice given in vomiting and dysentery	2.9
7.	*Cassia fistula*/ Amaltas	Caesalpiniaceae	in ringworms, in fungal infection, in chest and heart diseases, laxative for constipation	0.7
8.	*Cedrus deodara*/ Diyar	Pinaceae	In pulmonary disorders	-0.3
9.	*Cynoglossum zeylanicum*	Boraginaceae	In wound healing	2.2
10.	*Debregeasia longifolia*	Urticaeae		-0.9
11.	*Erigeron bellidioides*	Asteraceae		1.6
12.	*Galinsoga parviflora*	Asteraceae		3.0
13.	*Girardinia diversifolia*/ Badi bichhoo	Urticaceae	In treatment of ringworms, skin inflammatory problems, gastric problems	6.0
14.	*Hedychium spicatum*/ Jungli haldi	Zingiberaceae	In gastric problems	7.6
15.	*Malva rotundifolia*	Malvaceae		0.2
16.	*Nicandra physaloides*	Solanaceae		3.6
17.	*Onychium japonicum*	Pteridaceae		1.8
18.	*Oxalis corniculata*/ Khatti meethi bel	Oxalidaceae	Blood purifier, in scurvy problems	-9.4
19.	*Pinus roxburghii*/ Chir	Pinaceae	In snake bites and ulcers	-1.7
20.	*Pinus wallichiana*/ Kaayl	Pinaceae		0.7
21.	*Polystichum aculeatum*	Dryopteridaceae		-1.6
22.	*Potentilla fulgens*/ Bajra danti	Rosaceae	In teeth problems, in diarrhea problems	2.9
23.	*Pteris cretica*	Pteridaceae		2.5
24.	*Quercus leucotrichophora*/ Ban	Fagaceae	In dysentery and diarrheal problems	-5.9
25.	*Rhododendron arboretum*/ Buraansh	Ericaceae	In headaches, blood purifier, in gastric problems	4.3
26.	*Rosa sericea*/ Jungli gulab	Rosaceae	Ornamental	2.8
27.	*Rubia cordifolia*/ Manjith	Rubiaceae	Tonic, astringent, antidysenteric	1.3
28.	*Rubus ellipticus*	Rosaceae	In abdominal pains	3.1
29.	*Rumex hastatus*	Polygonaceae	Plant juice is used in dysentery, in throat aches, wound healing, astringent	-4.2
30.	*Rumex nepalensis*/ Jungli palak	Polygonaceae		-3.9
31.	*Salvia coccinea*/ Chhoti tulsi	Lamiaceae		0.3
32.	*Sonchus asper*	Asteraceae	Wound healing and antiseptic	-2.7
33.	*Thalictrum foliolosum*/ Pilizari	Ranunculaceae	In fever to lower body temperature, in skin problems, in snake bites, purgative	1.8
34.	*Urtica dioica/ Bichhoo ghaas*	Urticaceae	In joints problems, in jaundice, diuretic, antihelminthic, blood purifier.	23.2
35.	*Vinca major*	Apocynaceae		28.4
36.	*Viola biflora*	Violaceae		2.7

[#]Some plants were not known to local communities.
[†]Some plants were not observed to be in traditional uses.
*Negative lipase inhibition (LI) values demonstrate that lipase activity was observed to be increased with some plant extracts.

Table 2: Antilipase activities of *Vinca major* tissue extracts.

Scientific name	Family name	Part used	Lipase	IC_{50} value (µg/ml)
Vinca major	Apocynaceae	Leaf	PPL	1027.0
			Lipolase	297.0
			Steapsin	684.0
		Flower	PPL	403.0
			Lipolase	454.0
			Steapsin	409.0
Orlistat			PPL	9.5
			Lipolase	145.0
			Steapsin	142.0

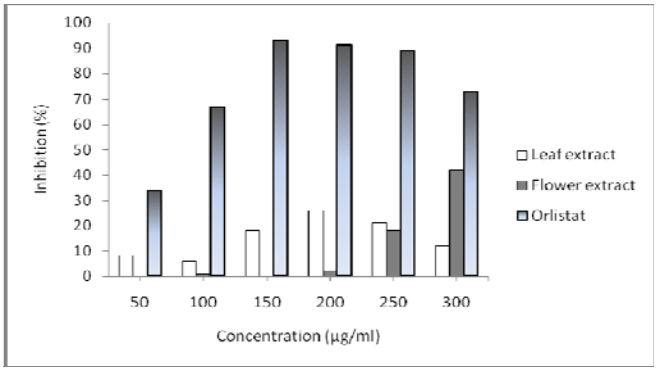

Fig 1: Inhibitory potential of tissue extracts of *Vinca major* and Orlistat towards PPL.

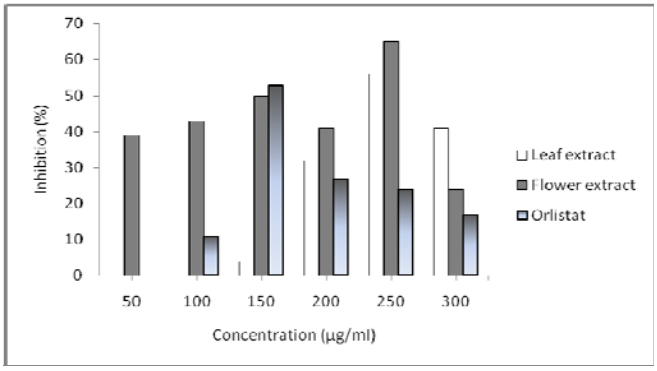

Fig 2: Inhibitory potential of tissue extracts of *Vinca major* and Orlistat towards Lipolase.

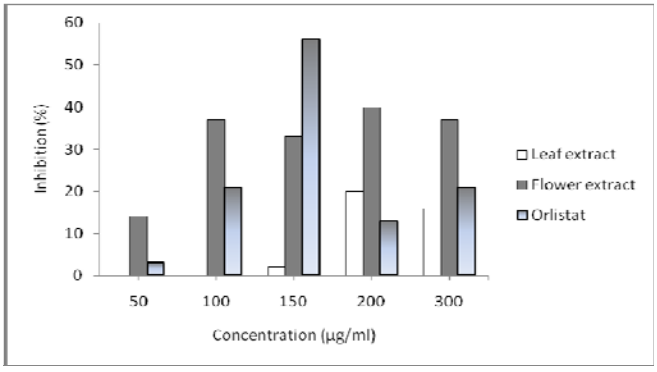

Fig 3: Inhibitory potential of tissue extracts of *Vinca major* and Orlistat towards Steapsin.

3.2 Kinetic analysis of lipase inhibition by VFE

The kinetics of inhibition mode of VFE was analyzed by double-reciprocal Lineweaver-Burk plot (Figure 4). From recorded results enzyme kinetics demonstrated that *Vinca major* might have mixed inhibition on PPL (Table 3). Further, the VFE reduced the turnover number (K_{cat}) of PPL that means the VFE retarded the activity of the PPL by binding to enzyme-substrate complex. Kinetic analyses have been done for many antilipolytic compounds/ extracts. The Orlistat was shown to be irreversible inhibitor of pancreatic lipase [30]. Platycodin D and tea saponins inhibited pancreatic lipase in competitive manner [31, 32] which is in contrast to our results. However, in accordance with our results some kinetic studies reported mixed inhibition on pancreatic lipase [33]. A mixed inhibitor binds at a distinct site from the active site but it can binds to the enzyme or enzyme substrate complex as well. It affects the K_m and V_{max} of the reaction. Components of VFE showed mixed type of inhibition as it could bind to enzyme or enzyme substrate complex [ES] and retarded its activity.

3.3 Anti-proliferatic effects of VFE

Cancer is an increasing health concern this time worldwide and there is an essence need to develop safer anti-cancerous therapeutics. Several natural extracts have been investigated for anti-proliferatic function on tumor cells for the same [34]. To address the anti-proliferatic potential of VFE, Hep-2 cells (human laryngeal carcinoma) were treated with different concentrations of VFE and assayed for viability using MTT. VFE reduced approximately 30% viability with IC_{50} (concentration at which fifty percent cell viability remains) 228 µg/ml when examined up to 150 µg/ml (Table 4).

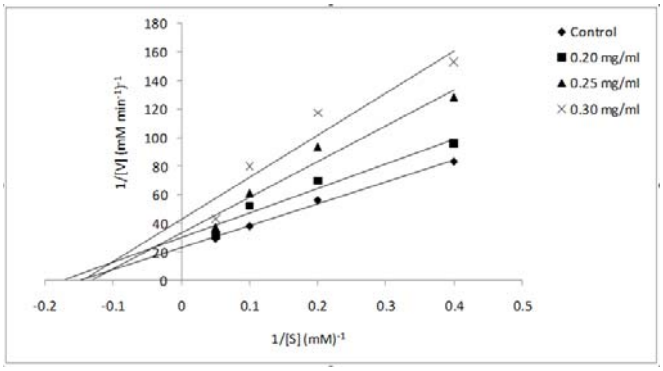

Fig 4: The Lineweaver-Burk plot of kinetic analysis for PPL at three different concentrations of *Vinca* flower extracts (0.20, 0.25 and 0.30 mg ml^{-1}) in the presence of four different *p*-NPP concentrations.

Table 3: Kinetic analysis of PPL at varying concentrations of *p*-NPP and *Vinca* flower extract.

[I] mg ml^{-1}	Velocity of PPL activity in different concentrations of substrate [S] (mM)				V_{max} (mM min^{-1})	K_m (mM)	K_{cat} (S^{-1})	K_{cat}/K_m (M^{-1}S^{-1})
	2.5	5	10	20				
0	0.012	0.017	0.026	0.034	0.043	6.65	997.17	1.49x 10^5
0.20	0.010	0.014	0.019	0.031	0.033	5.81	765.27	1.31x 10^5
0.25	0.007	0.010	0.016	0.026	0.029	7.41	672.51	0.9x 10^5
0.30	0.006	0.008	0.012	0.023	0.023	6.82	533.37	0.78x 10^5

Table 4: Cytotoxicity of *Vinca major* flower extracts against Hep-2C cell line.

S. No.	Concentration (µg/ml)	% Cells inhibition ± SD
1.	10	-
2.	30	-
3.	50	5.80 ± 3.68
4.	70	8.81 ± 5.75
5.	90	7.52 ± 3.93
6.	110	30.74 ± 14.81
7.	130	31.50 ± 17.29
8.	150	26.02 ± 8.39

3.4 Phytochemical constituents of *Vinca major*

Phenols, alkaloids, flavonoids and glycosides were observed to be present in *Vinca* flowers extracts (Table 5). High quantity of flavonoids and alkaloids were found (Table 6) in both VFE (1000 and 1520 mg/100 g) and VLE (5100 and 1420 mg/100 g), respectively.

Table 5: Qualitative analysis of phytochemicals in methanolic *Vinca* flower extracts. The presence of component is indicated by + sign and absence is indicated by a – sign.

S. No.	Phytochemical	Result
1.	Phenols and tannins	+
2.	Flavonoids	+
3.	Saponins	+
4.	Alkaloids	+
5.	Terpenoids	-
6.	Glycosides	+
7.	Steroids	-
8.	Proteins	-
9.	Carbohydrates	-
10.	Carbonyls	-

Table 6: Major phytochemical constituents of *Vinca major* tissue extracts.

Phytochemicals	Amount (mg/ 100 g)	
	VFE	VLE
Total phenolics	1830.0	850.0
Tannins	850.0	17.0
Flavonoids	**2320.0**	1420.0
Alkaloids	1000.0	**5100.0**
Saponins	1520.0	1600.0

Phytochemicals are known for their pharmaceutical/ biological actions [35]. Currently, a number of synthetic antioxidants are available but generally there is still a great demand to find safer antioxidant/ anti-ageing pharmaceutical principals. Flavonoids and polyphenols are known to suppress reactive oxygen species and free radicals [36]. Besides flavonoids, presence of high amount of alkaloids in VLE was a surprising result of our study. Thus it is quite evident that alkaloids might be responsible for antioxidant property of VLE. Saponins have been known for their hypolipidemic and hypocholesterolemic activities [37]. The recorded results in the present study indicated that VFE possessed saponins in sufficient amounts. But additional sufficient flavonoids and phenolic content also can't be ignored. They may also have some critical role for antilipolytic activity.

3.5 Antiradical activities of *Vinca major*

3.5.1 DPPH radical scavenging activity

Hydrogen peroxide and superoxide can interact in the presence of certain transition metal ions to yield a highly-reactive oxidizing species, the hydroxyl radical. The antioxidants react with the stable free radical DPPH (deep violet color) and convert it to 1, 1-diphenyl-2-picryl hydrazine with decolouration. The method is based upon the reduction of methanol DPPH solution in the presence of hydrogen donating antioxidant due to formation of the non radical form DPPH-H by the reaction [38]. Assay showed that VLE eliminated DPPH radical comparatively much efficiently than VFE in a dose dependent manner (Figure 5). IC_{50} values for VLE, VFE and ascorbic acid were 170, 353 and 91, µg/ml respectively.

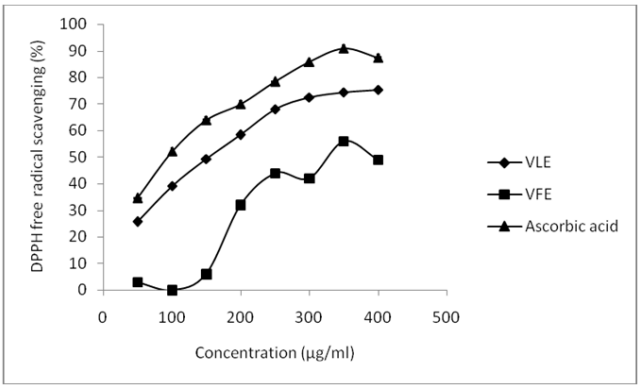

Fig 5: DPPH scavenging by *Vinca major* tissue extracts and ascorbic acid.

3.5.2 H_2O_2 radical scavenging activity

VLE (IC_{50} value 600 µg/ml) and VFE (IC_{50} value 698 µg/ml) were found to be less potent in scavenging H_2O_2 radicals when compared to ascorbic acid (IC_{50} value 301 µg/ml) (Figure 6). H_2O_2 itself is not very reactive, but it can either sometimes be toxic to the cell because it may give rise to hydroxyl radical in the cells through the Fenton reaction or it may oxidize the enzyme molecules too thus

rendering them biologically less active. Thus, quick removal of H_2O_2 is very important for protection of food systems [39].

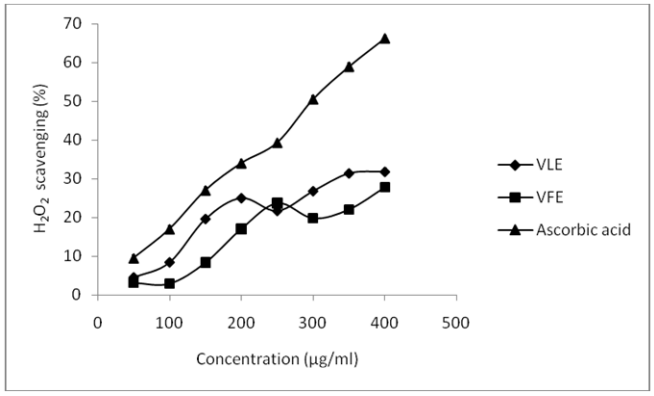

Fig 6: Hydrogen peroxide scavenging by *Vinca major* tissue extracts and ascorbic acid.

3.5.3 Hydroxyl radical scavenging assay

Hydroxyl radicals are the major active oxygen species that cause lipid oxidation and subsequent enormous biological damage. Hydroxyl radical scavenging activity of VLE and VFE were measured. Ferric EDTA was incubated with H_2O_2 and Ascorbic acid; hydroxyl radicals were formed in free solution and were detected by their ability to degrade deoxyribose into fragments that on heating with TBA and form a pink product [40]. The percentage of hydroxyl radical scavenging increased with an increasing concentration of VLE while VFE did not prove to be a good hydroxyl radical scavenger. The IC_{50} values of VLE and ascorbic acid were 611 µg/ml and 445 µg/ml, respectively (Figure 7).

3.5.4 Iron reducing power assay

Reducing power assay measures the electron donating capacity of an antioxidant. The reduction of the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) is measured by the intensity of the resultant blue-green solution which absorbs at 700 nm, and an increased absorbance is indicative of higher reducing power [41]. However, reducing power of the *Vinca major* (VLE and VFE) and ascorbic acid increased gradually over the tested concentration range studied but VLE (50% iron reduction at 338 µg/ml) and VFE (50% iron reduction at 751 µg/ml) were found to be less potent when compared to ascorbic acid (50% iron reduction at 165 µg/ml) (Figure 8). These findings suggested that the *Vinca major* leaf extract is capable of donating electrons and could therefore react with free radicals or terminate chain reactions.

3.5.5 Superoxide radical scavenging activity

The VLE (300 µg/ml) as well as ascorbic acid strongly inhibited the superoxide radical generation. Percentage inhibition of superoxide radical generation by VLE, VFE and ascorbic acid gradually increased in a dose-dependent manner showing the IC_{50} value of 421, 1360 and 311 µg/ml, respectively (Figure 9).

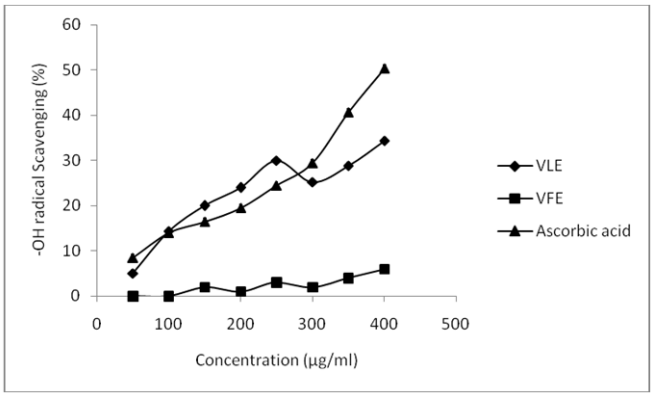

Fig 7: Hydroxyl radical scavenging by *Vinca major* and ascorbic acid.

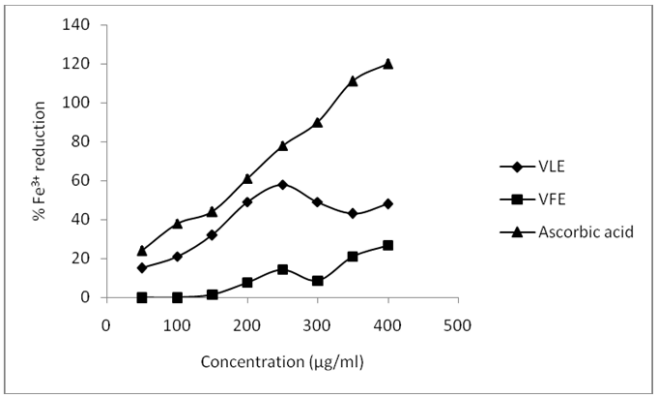

Fig 8: Iron reducing power of *Vinca major* tissue extracts and ascorbic acid.

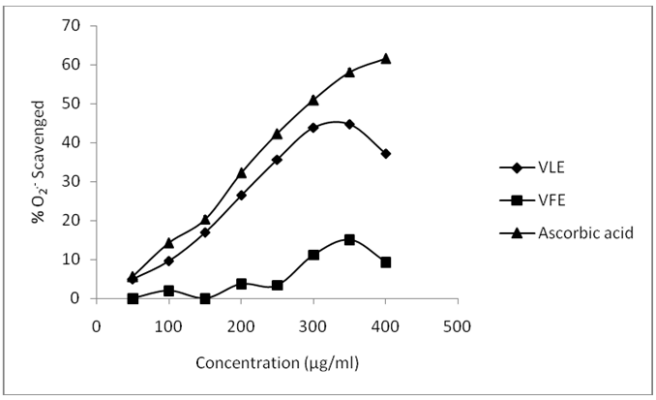

Fig 9: Superoxide radical scavenging by *Vinca major* tissue extracts and ascorbic acid.

3.5.6 Hemolytic effects of *Vinca major* flower extract

Hemolytic activity of the VFE was measured against normal human erythrocytes. Interestingly, the VFE preparation of *Vinca major* possessed mild hemolytic activity with an IC_{50} value 2406 µg/ml. Hemolytic activity was found to be somewhat increasing with an increase in amount of VFE (Figure 10). It is quite possible that presence of saponins in VFE may have some membrane damaging effects on the human RBCs leading to their hemolysis *albeit* to a little extent as reported recently [42].

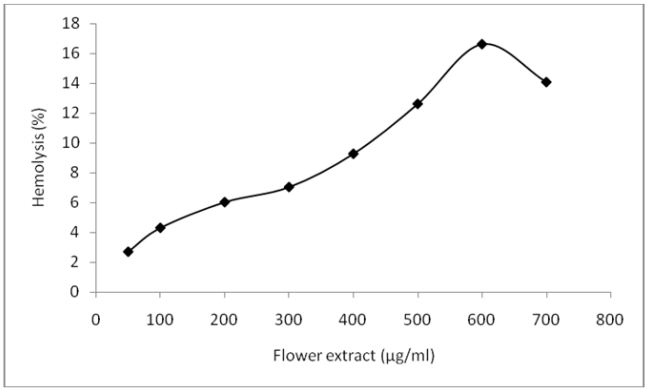

Fig 10: Hemolytic activity of *Vinca major* flower extract (VFE) towards human RBC.

3.5.7 Significance of the study

Flower and leaf tissue extracts of *Vinca major* appeared to be good sources for antilipolytic and antioxidant properties respectively. Additionally, *Vinca major* inhibited the growth of carcinoma cells also. However, antilipase potential of *Vinca* flower extract is comparatively lesser than the discovered lipase inhibitory molecules, but it is suitable to address out the mechanism of lipase inhibition. Moreover, studies are needed to confirm these results at molecular level and molecular characterization of active constituents of *Vinca major* will be achieved by fractionation on suitable matrices followed by structural elucidation from NMR/ Mass spectrometry etc. Further, *in vivo* studies will be done on a mouse animal model to confirm these results. An animal model will definitely serve as a foundation for comprehensive and safer therapeutic strategies for the management of obesity and other related diseases.

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