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Original Article

Evaluation of *in vitro* Cholesterol Esterase Inhibitory Activity of Few Selected Vegetables – An Indication of Cardioprotective Property

Thiyagarajan Sathishkumar, Muruvanda Bhuvan Uthaiah*, Ravichandran Vinu Varshith, Kuppamuthu Kumaresan, Vinohar Stephen Rapheal

ABSTRACT

Background: Cardiovascular diseases are responsible for severe mortality rate amongst the population. Several drugs available to control and/ or cure the same, cause severe adverse effects amidst human population. Contextually, the present study was devised to screen the *in vitro* cholesterol esterase (CEase) activity of few selected vegetables as an indicator of cardioprotective property. **Objectives:** To screen and purify the polyphenolic fractionate from the vegetable(s) extract that record an optimal CEase activity. **Materials and Methods:** CEase inhibitory activity was investigated using standard spectrophotometric assay. Microwave assisted central composite design based response surface methodology (CCD based RSM) was executed to quantify an improvement in the CEase inhibitory activity of *Solanum melongena* extract. One dimensional thin layer chromatography (1D TLC) and two-dimensional preparative thin layer chromatography (2D PTLC) analyses were carried out for *Brassica oleracea var. botrytis* hot water **e**xtract to identify and eluate the polyphenolic fractionate, respectively. **Results:** Significant CEase inhibitory activity was noticed in the hot water extract of *Brassica oleracea var. botrytis* (84.65 ± 3.29%) than the other vegetable extracts. Even though the optimal process model was validated (at 5% level), no significant improvement was recorded in the CEase inhibition. The presence of polyphenolic content in the hot water extract of *Brassica oleracea var. botrytis* (R_f = 0.69) was confirmed by comparing with standard quercetin (R_f = 0.60). Strong blue and moderate brownish yellow color spots revealed the presence of phenolic acid and flavonoid eluates respectively, were purified using 2D PTLC technique. The eluate has recorded a near moderate (28.57 ± 2.12%) CEase inhibitory activity. **Conclusion:** *Brassica oleracea var. botrytis* possess significant CEase inhibitory activity and proved to be a good diet to protect from various cardiovascular diseases.

Key words: *Brassica oleracea var. botrytis,* Cholesterol esterase, *Solanum melongena,* Microwave Assisted Response Surface Methodology, Flavonoid.

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INTRODUCTION

Cardiovascular Diseases (CVDs) that comprised of ischemic heart disease, coronary heart disease, myocardial infarction, cerebrovascular disease, hypertensive heart disease, atherosclerosis, rheumatic heart disease, deep vein thrombosis and pulmonary embolism belongs to noncommunicable disease category, and leads to a very high mortality rate, disability and also, create a significant increase in the socio-economic burden amongst human population.[1] According to World Health Organization (WHO), India has recorded one fifth of CVDs related deaths globally, that is equivalent to 272 per 100000 population.[2] The major conventional etiological risk factors of CVDs include tobacco utility, unhealthy diet, physical inactivity, ageing, hypertension, diabetes, obesity, hyperlipidemia and familial predisposition.[3-5] In India, states such as Punjab, Kerala and Tamil Nadu have recorded a greater number of CVDs cases, and also recorded high prevalence of increased blood pressure and blood cholesterol levels which probably could be the rationale for the contract of CVDs. Henceforth, an imbalance in the blood cholesterol level should be very carefully avoided through follow up of appropriate food style and lifestyle.[6]

There are several types of hypolipidemic as well as hypocholesterolemic drugs such as statins, bile aid sequestrants, niacin and orlistat with different mechanism of action are available in the market. Statins can lower the serum LDL level,[7] niacin can reduce both serum triglycerides and LDL,[8] bile acid sequestrants can lower serum LDL level and

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elevate HDL level,[9] and orlistat can effectively prevent the absorption of lipids through inhibition of pancreatic lipase.[10] Even though different strategies are adopted very effectively to prevent an increase in the triglyceride and cholesterol levels, severe adverse effects such as muscle damage, rhabdomyolysis, hepatotoxicity, gastrotoxicity, insulin resistance and hyperglycemia are documented by the above said drugs. Henceforth, isolation and identification of efficacy oriented alternative nontoxic drugs from plant species are considered as significant therapeutical by the scientific community.

India is rich in flora and have a widespread of medicinal plants across the country. Traditionally, whole parts or a particular part of a plant was used to control or cure various diseases and the main rationale for their utilization is due to mild or nil toxicity as well as nil adverse effects. Phytochemicals or secondary metabolites that are significantly distributed in the plants are responsible to execute diversified pharmacological activities.[11] Previous studies related to the assessment of *in vitro* as well as *in vivo* cardioprotective potential of the medicinal plants have revealed its pivotal role in the drastic reduction of risk of cardiovascular diseases and its associated complications.[12-15]

In this context, six different vegetables [(*Brassica oleracea var. capitata* (cabbage), *Raphanus sativus* (radish), *Solanum melanogena* (brinjal), *Moringa oleifera* (drumstick), *Trichoxanthes cucumerina* (snakegourd), and *Brassica oleracea var. botrytis* (cauliflower)] were selected in a random manner for the present study. The selected vegetables have recorded significant antioxidant, free radical scavenging, inhibition against α-glucosidase, and ACE 1, cardio and hepatoprotective, antimicrobial, antiviral, antimutagenic, antidiabetic, anticancer, anti-inflammatory, antihypertensive, hypocholesterolemic and hypolipidemic properties.[16-26] Eventhough substantial documentation for numerous medicinal properties on the selected vegetables is available, no report is observed on the cholesterol esterase inhibitory activity and hence, our laboratory has focussed on the same to document the probable inhibitory activity of the above slated enzyme as a promising candidate to control the contract of several cardiovascular diseases.

MATERIALS AND METHODS

Chemicals

para-nitrophenyl butyrate (pNPB) (Sigma Aldrich, USA) and cholesterol esterase (Crest biosystems, Goa). All the other chemicals and solvents were purchased from Merck India Pvt. Ltd. and S.D. Fine Chem Ltd.

Collection and processing of vegetables

Fresh vegetables such as *Brassica oleracea var. capitata* (Cabbage), *Raphanus sativus* (Radish), *Solanum melanogena* (Brinjal), *Moringa oleifera* (Drumstick), *Trichosanthes cucumerina* (Snakegourd) and *Brassica oleracea var. botrytis* (Cauliflower) were purchased from the local market situated at Coimbatore, Tamil Nadu, India during the month of September. The vegetables were washed with tap water followed by distilled water until the dirt was removed and the edible part was used for the extraction process.

Preparation of hot water extract (HWE)

About 5 g of fresh sliced vegetable, added 50 ml of distilled water in a clean beaker and kept in a boiling water bath at 90°C for five minutes. The resultant extract was cooled, filtered using Whatman no. 1 filter paper, and added 25% ammonium sulphate to precipitate the protein content. Furthermore, the extract was centrifuged at 10,000 rpm for 10 min to sediment the protein content completely and the resultant supernatant was used for experimental analysis.[27]

Preparation of pressurized hot water extract (PHWE)

In a clean dry vessel, added 5 g of fresh sliced vegetable and 50 ml of distilled water. The content was extracted in an oil bath at 180°C 10.027 barr for 15 min.[28] The resultant extract was cooled, filtered using Whatman no. 1 filter paper, and added 25% ammonium sulphate to precipitate the protein content. Furthermore, the extract was centrifuged at 10,000 rpm for 10 min to sediment the protein content completely and the resultant supernatant was used for experimental analysis.

Preparation of organic solvent extract (OSE)

In a clean dry 250 ml conical flask, 5 g of the fresh sliced vegetable and 50 ml of ethanol were added and kept in an orbital shaker for overnight (12 – 16 hr) at room temperature. The extract was then cooled, filtered using Whatman no. 1 filter paper, and added 25% ammonium sulphate to precipitate the protein content. Furthermore, the extract was centrifuged at 10,000 rpm for 10 min to sediment the protein content completely and the resultant supernatant was used for experimental analysis. The same protocol was repeated using acetone.[29]

In vitro cholesterol esterase inhibitory assay

A method described by Kumar *et al*., (2011) was adopted to investigate the *in vitro* cholesterol esterase inhibitory activity. In a clean dry test tube ("test"), pipetted 0.5 ml phosphate buffer solution (pH 7.0), 0.5 ml pNPB (200μM), 0.5 ml test sample and mixed well. To the resultant mixture, added 0.5 ml cholesterol esterase, incubated at room temperature for 5 min and the formed yellow color *p*-nitrophenol was measured spectrophotometrically at 405 nm.[30] Simultaneously, a "control" was performed without the addition of test sample. The CEase inhibition was calculated as follows: CEase inhibition (%) = [(Activity of control – activity of test)/ activity of control] × 100.

Estimation of total phenolic content (TPC) by Folin – Ciocalteau method

A modified method described by Singleton and Rossi (1965) was used to quantify the TPC in all the extracts. Added 0.5 ml of the extract, 5.5 ml of distilled water and 0.5 ml of Folin-Ciocalteau reagent, and incubated at room temperature for 3 min. To the resultant mixture, added 2 ml of 7% sodium carbonate and kept in a boiling water bath for about a minute. The blue colour formed was read spectrophotometrically against a blank at 650 nm. Gallic acid was used as a standard to construct a calibration curve.[31]

Two-dimensional thin layer chromatography (2D TLC) and preparative thin layer chromatography (2D PTLC) analyses

A glass plate (20 × 20 cm^2) was coated with silica gel slurry (0.2 mm thick) and was dried at room temperature for few minutes. The dried plate was activated at 100°C for about 30 min and then kept at 50°C overnight in a hot air oven. The sample extract and standard marker (quercetin) was spotted individually 2 cm apart from the bottom edge of the plate using capillary tube. The first dimensional chromatogram was developed by keeping the glass plate in a saturated glass chamber that contain the mobile phase (petroleum ether: acetone: formic acid [35:10:5]) and the plate was rotated to 180° clockwise to develop a second-dimension chromatogram using another type of mobile phase (ethyl acetate: glacial acetic acid: formic acid: distilled water [100:11:11:26]). The developed plate was air dried and visualized under far UV light after spraying with liquid ammonia for the identification of polyphenols.

The same procedure was followed for two-dimensional preparative thin layer chromatography (2D PTLC) technique with a change in the

thickness (2 mm) of the silica gel slurry. The polyphenolic fractionate (emit different colors) spotted under far UV light was eluted, added adequate volume of phosphate buffer (pH 7.0) and centrifuged till a clear supernatant was obtained. The supernatant was lyophilized and used for further experimental analysis.[32]

Microwave assisted extraction (MAE) process optimization of CEase inhibitory activity in *Solanum melanogena* using central composite design (CCD) based response surface methodology (RSM)

Microwave assisted extraction (MAE) optimization process was adopted to find or observe the possibility of an improved the CEase inhibitory property in *Solanum melanogena*. Three different independent variables viz., power (watt), material ratio (w/v) and time duration (seconds) (Table 1) were selected to record the dependent variable (CEase inhibition) response. The response can be validated using a quadratic polynomial model (Eq. 1),

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\Sigma\beta_{ij} x_{ij} \quad (1)$$

Where, "Y" is the response/ dependant variable (CEase inhibition) measured against each combination of independent variables at various factorial level. Similarly, β_0, βi, βii, and βij in the equation denotes different regression coefficients such as intercept, linearity, square, and interaction, respectively, and x_i, x_j and x_k were codes of the independent variables. The coefficients of the second order polynomial equation were obtained through multiple regression equation. The degree of fitness of the model was validated using correlation coefficient (R^2), adjusted correlation coefficient (R^2adj), p-value and lack-of-fit. F-test and t-test were used to check the statistical significance of R^2 and the regression coefficients, respectively.[33] Moreover, surface and contour plots were predicted to interpret the optimal condition of the selected variables.

Table 1: Design of Response Surface Methodology (RSM).

Std order	Power (watt)	Time duration (seconds)	Material ratio (w/v)
1	360	15	1:10
2	720	15	1:10
3	360	45	1:10
4	720	45	1:10
5	360	15	1:20
6	720	15	1:20
7	360	45	1:20
8	720	45	1:20
9	237.28	30	1:15
10	842.72	30	1:15
11	540	4.77	1:15
12	540	55.23	1:15
13	540	30	1:6.59
14	540	30	1:23.41
15	540	30	1:15
16	540	30	1:15
17	540	30	1:15
18	540	30	1:15
19	540	`	1:15
20	540	30	1:15

Statistical analyses

All the experiments were performed in triplicates and the results were expressed as mean ± s.d. Karl Pearson correlation coefficient, Student's *t*-test and one way ANOVA were calculated using office 365 excel. CCD based RSM was formulated and analyzed using Design Expert 7.0.0 trial version.

RESULTS AND DISCUSSION

In vitro CEase inhibitory activity of different vegetable extracts

The results of *in vitro* cholesterol esterase inhibitory activity and total polyphenolic content of the vegetable extracts were depicted in Table 2. Hot water extract (HWE) of *Brassica oleracea var. botrytis* has recorded the highest CEase inhibition (84.65± 3.29%) than the other vegetable extracts. The one-way ANOVA (Table 3) has showed that HWE was superior and significant (p = 0.021) at 5% level than the other techniques. Similarly, an appreciable polyphenolic content was observed in all the extracts. It was found that PHWE showed some promising CEase inhibitory effect on par with HWE, whereas, OSE (ethanol and acetone) were found to be very poor. The CEase activity exhibited by different vegetables were depicted in descending manner as followed: *Brassica oleracea var. botrytis* (HWE: 84.65 ± 3.29) > *Brassica oleracea var. capitata* (PHWE: 74.19 ± 2.98) > *Moringa oleifera* (PHWE: 70.76 ± 3.53) > *Solanum melongena* (OSE: 57.22 ± 3.04) > *Raphanus sativus* (HWE: 49.88 ± 2.21) > *Trichosanthes cucumerina* (PHWE: 14.28 ± 4.27). Previous reports have documented an appreciable pancreatic lipase activity of few vegetables such as *Solanum melongena*,[34] *Raphanus sativus*,[35] *Moringa oleifera*,[36] and very poor activity for *Brassica oleracea var. botrytis* and *Brassica oleracea var. capitate*,[37] and no documentation for *Trichosanthes cucumerina*. So far, no scientific documentation is available for CEase inhibitory property of the selected vegetables and our studies have proved that both the *Brassica oleracea var. botrytis* and *Brassica oleracea var. capitata* possess the same.

Investigation of cholesterol esterase inhibitory property of Solanum melongena through MAE assisted CCD based RSM

Even though *Brassica* variety vegetables have exhibited significant CEase inhibitory effect, our study has revealed that *Solanum melongena* and *Trichosanthes cucumerina* possess very poor CEase inhibition than the other vegetables. Moreover, *Trichosanthes cucumerina* was too poor than *Solanum melongena* in exhibiting CEase inhibitory activity and we felt that a significant improvement for the same may not be expected or impossible. Hence, microwave assisted CCD based RSM process optimization was adopted to improve the CEase inhibitory activity of *Solanum melongena*, and independent variables such as power (watt), material ratio (g/ ml) time duration (seconds) were selected, experiments were performed, and analyzed the expected response (CEase inhibition [%]). The result of the same was showed under Table 4a.

Amongst twenty various experimental runs performed, an optimal condition (Exp. No. 10) of 842.72 (watt), 30 (sec) and 1:15 (g/ ml) was found to record a maximal CEase inhibition (64.7 ± 2.59). When compared to HWE and OSE (ethanol) extraction methods, about 3.25 and 1.13 fold increase of CEase inhibition was noticed. The actual multiple regression equation generated based on second order polynomial equation was expressed as:

Table 2: Comparative results of CEse inhibitory activity of various vegetable extracts.

S. No	Name of the vegetables	Hot water		Pressurised hot water		Ethanol		Acetone	
		CEase inhibition (%)	Polyphenolic content (mg/ g tissue)	CEase inhibition (%)	Polyphenolic content (mg/ g tissue)	CEase inhibition (%)	Polyphenolic content (mg/ g tissue)	CEase inhibition (%)	Polyphenolic content (mg/ g tissue)
1.	Brassica oleracea var. capitata	72.24 ± 1.83	0.3 ± 0.01	74.19 ± 2.98	2.25 ± 0.01	65.43 ± 2.57	0.32 ± 0.0	10.69 ± 1.62	0.54 ± 0.02
2.	Raphanus sativus	49.88 ± 2.21	0.31 ± 0.08	44.32 ± 4.94	3.5 ± 0.01	34.69 ± 1.73	1.77 ± 0.19	3.54 ± 2.11	0.3 ± 0.16
3.	Moringa oleifera	44.94 ± 0.59	0.34 ± 0.1	70.76 ± 3.53	2.33 ± 0.12	44.07 ± 5.94	0.05 ± 0.02	2.05 ± 1.33	0.93 ± 0.17
4.	Brassica oleracea var. botrytis	84.65 ± 3.29	0.45 ± 0.16	42.85 ± 5.21	1.18 ± 0.07	11.97 ± 0.83	0.39 ± 0.12	4.87 ± 1.44	0.39 ± 0.06
5.	Trichosanthes cucumerina	8.1 ± 5.85	0.09 ± 0.05	14.28 ± 4.27	1.44 ± 0.01	5.28 ± 2.77	0.09 ± 0.04	3.29 ± 5.70	0.1 ± 0.03
6.	Solanum melongena	19.88 ± 1.02	0.52 ± 0.12	17.92 ± 2.30	3.54 ± 0.12	57.22 ± 3.04	0.54 ± 0.02	10.06 ± 3.45	1.44 ± 0.18

Table 3: Results of one-way ANOVA between different extraction methodologies.

Groups	Count	Sum	Average	Variance		
Hot water	6	279.69	46.615	862.9866		
Pressurised hot water	6	264.32	44.05333	638.4773		
Ethanol based	6	218.66	36.44333	580.6469		
Acetone based	6	34.5	5.75	13.67596		
Source of Variation	**SS**	**d_f**	**MS**	***F***	***P*-value**	**F crit**
Between Groups	6370.667	3	2123.556	4.053	0.021078	3.098391
Within Groups	10478.93	20	523.9467			
Total	16849.6	23				

Table 4a: CEase inhibitory activity of HWE of *Solanum melongena* optimized using MAE assisted CCD based RSM.

Std order	Power (Watt)	Time duration (sec)	Material ratio (g/ ml)	CEase inhibition (%)	
				Experimental	Predicted
1	360	15	1:10	15.39 ± 4.63	8.15
2	720	15	1:10	41.94 ± 3.61	37.85
3	360	45	1:10	19.48 ± 2.43	5.15
4	720	45	1:10	29.41 ± 3.22	18.65
5	360	15	1:20	3.59 ± 2.17	2.25
6	720	15	1:20	50.005 ± 5.80	53.55
7	360	45	1:20	24.61 ± 1.14	17.25
8	720	45	1:20	55.88 ± 1.24	52.35
9	237.28	30	1:15	3.59 ± 0.66	9.58
10	842.72	30	1:15	64.7 ± 2.59	64.07
11	540	4.77	1:15	10.25 ± 4.99	16.70
12	540	55.23	1:15	18.98 ± 3.32	13.16
13	540	30	1:6.59	3.08 ± 1.24	10.31
14	540	30	1:23.41	35.3 ± 2.02	33.69
15	540	30	1:15	43.94 ± 2.00	27.66
16	540	30	1:15	12.31 ± 2.93	27.66
17	540	30	1:15	29.41 ± 1.67	27.66
18	540	30	1:15	38.24 ± 3.08	27.66
19	540	30	1:15	6.16 ± 2.56	27.66
20	540	30	1:15	47.06 ± 3.34	27.66

Table 4b: One way ANOVA of the predicted quadratic model of the selected response (CEase inhibitory activity).

Source	Sum of Squares	d_f	Mean Square	*F*-value	*p*-value	
Model	4953.29	9	550.37	3.05	0.0484	significant
A-Power	3446.09	1	3446.09	19.12	0.0014	
B-Time duration	80.40	1	80.40	0.4462	0.5193	
C-Material ratio	492.98	1	492.98	2.74	0.1291	
AB	126.13	1	126.13	0.6999	0.4224	
AC	212.23	1	212.23	1.18	0.3033	
BC	156.07	1	156.07	0.8661	0.3740	
A^2	155.25	1	155.25	0.8615	0.3752	
B^2	189.13	1	189.13	1.05	0.3298	
C^2	57.94	1	57.94	0.3215	0.5832	
Residual	1802.08	10	180.21			
Lack of Fit	368.57	5	73.71	0.2571	0.9188	not significant
Pure Error	1433.51	5	286.70			
Cor Total	6755.37	19				

$$Y\ [\text{CEase inhibition (\%)}] = 4.77 - 0.063\ x_1 + 1.04\ x_2 - 1.25\ x_3 - 0.0015\ x_1x_2 + 0.006\ x_1x_3 + 0.06\ x_2x_3 + 0.0001\ x_1^2 - 0.02\ x_2^2 - 0.08\ x_3^2$$

Paired Student's *t*-test (two tail) analysis between the experimental and predicted values were found to be non-significant at 5% level ($p = 0.342$) and existence of a good correlation ($R^2 = 0.841$) has proved the validity of the experiment. One way ANOVA of the quadratic model and the lack of fitness were found to be significant ($p = 0.0484$) and non-significant ($p = 0.9188$), respectively, has again proved the reliability of the predicted model (Table 4b). Moreover, power (watt) was found to influence the CEase inhibitory activity to a greater extent (significant at 1% level: [$p = 0.0014$]) followed by material ratio ($p = 0.129$) and time duration ($p = 0.159$).

CCD based RSM is a widely adopted software technique that is used to find an optimal condition for the selected response through certain independent variables. The software generates normal, residual, single factor, interactions, contour and surface plots, and regression equation and ANOVA based coefficients in order to validate the developed process model.[38] Furthermore, RSM saves time duration, lowers financial constraint and utilize less amount of raw material.

Amongst the different techniques, microwave assisted extraction is considered as a contemporary and advanced method for the extraction of phytoconstituents.[39] MAE is also successful in enhancing the yield of polyphenols such as phenolic acids, flavonoid, and tannins through adopting independent variables such as power, time duration, material ratio and type of solvent.[40,41] In this study, the variable power play a pivotal role in facilitating the CEase inhibitory activity. A gradual change of the red color (poor CEase inhibition) to green color (moderate CEase inhibition) and then to yellow color (high CEase inhibition) was observed in both the contour and surface plots (Figure 1(A, A1, B and B1)). A very poor inhibitory activity was recorded at low power (237.28 watt; 3.59 ± 0.66 %), and a gradual increase was observed at 540 watt (29.41 ± 1.67 %) and reached an optimal/ maximal inhibition at 842.72 watt (64.7 ± 2.59) (Figure 2(A)). When the output power was raised, an increase in the thermal energy (power is directly proportional to temperature) occurs that leads to the destabilization of covalent and non-covalent interactions of the cell wall and membrane, a decrease in the surface tension and viscosity of the solvent, and an increase in the kinetic energy/ diffusivity of the phytoconstituents into the cell free system,[42] which can elicit an enhanced CEase inhibition.

The next variable selected for the investigation of CEase inhibitory property was time duration. The depicted surface and contour plots in Figure 1(C and C1) clearly revealed a slight inverse effect of time duration against CEase inhibition. Initially, a poor response (10.25 ± 4.99) at 4.77 sec was noticed (yellowish green color at the bottom of both x- and y-axis), then slowly the inhibitory power reached an optimal level (intense yellow color) at 30 sec (64.7 ± 2.59) and recorded a declined activity (18.98 ± 3.32) at 55.23 sec (slight red color zone towards end of x-axis). Results of one factor analysis (Figure 2(B)) also supported the same. An increased CEase inhibitory activity recorded during the middle phase of the time duration was due to the rapid diffusion of the phytoconstituents into the solvent. Whereas a gradual decrement of the same by an increase in the time duration was probably due to an establishment of equilibrium in the diffusion process which follows Fick's second law and also owed to excess degradation of the phytoconstituents.[43]

The final and the last variable selected for the study was material ratio and here, water was adopted as an appropriate solvent due to its high dielectric constant (lead to better absorption of microwave energy), increased diffusion into the sample matrix and acceleration of mass transfer of the phytoconstituents.[44] The result was shown under

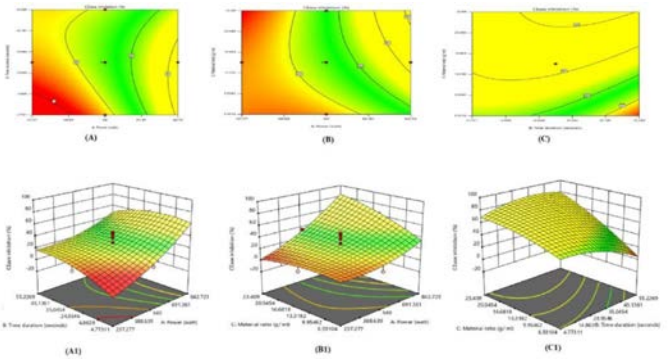

Figure 1: (A) Contour plot and (A1) Surface plot of output % cholesterol esterase inhibition vs. Power (W), Time (sec) (B) Contour plot and (B1) Surface plot of output % cholesterol esterase inhibition vs. Power (W), Material ratio (w/v). (C) Contour plot and (C1) Surface plot of output % cholesterol esterase inhibition vs. Time (sec), Material ratio (w/v).

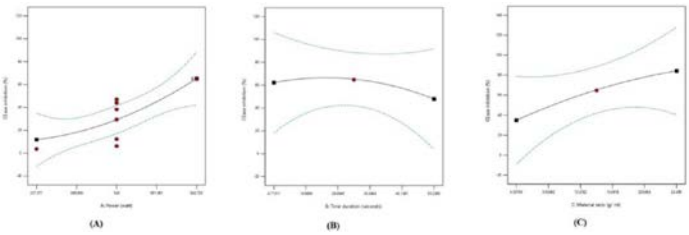

Figure 2: (A) Individual effect of Power against cholesterol esterase inhibitory activity (B) Individual effect of Time against cholesterol esterase inhibitory activity (C) Individual effect of Material ratio against cholesterol esterase inhibitory activity.

Figure 1(B and B1) and Figure 2(C). The red color at the bottom portion of y-axis (Figure 1(B)) revealed a poor inhibition of CEase activity (3.08 ± 1.24) recorded at 1:6.59, and the intensity of the color gradually faded, changed to yellow color at 1:15 that indicated an optimal CEase inhibitory activity (64.7 ± 2.59). It was further noticed a steady state kinetics of the response between 1:16 to 1:20 (Figure 2(C)) and, again, an appearance of intense red color was noticed at the top portion of y-axis at a ratio of 1:23.41 that denotes a drastic decrease in the inhibitory property (35.3 ± 2.02). In general, a concentrated material ratio (possess less amount of solvent) would record lesser response due to the charring effect of the material that leads to the severe loss of the phytoconstituents. Likewise, a diluted ratio may again lead to a decreased response owed to lesser convection of heat transfer into the material which results in decreased leaching of the phytoconstituents, and also, higher hydrolytic effect of the phytoconstituents,[45] that can cumulatively decreased the CEase inhibition.

Identification and isolation of Polyphenols of *Brassica oleracea var. botrytis* using 1D thin layer chromatography and 2D preparative thin layer chromatography techniques

The 1D TLC analysis of HWE of *Brassica oleracea var. botrytis* has revealed the presence of polyphenolic content ($R_f = 0.69$) that comprised of flavonoid (brownish yellow) and phenolic acid (blue color) moieties that was compared with a standard quercetin ($R_f = 0.60$) (Figure 3). Attempt was also made to eluate the identified polyphenols using 2D PTLC technique. Two strong polyphenolic spots (blue color and dull yellow color) were observed and eluted (Figure 4). The purified

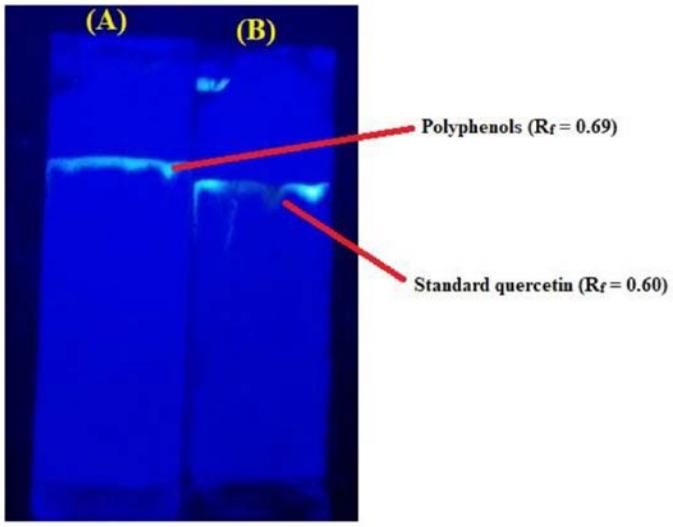

Figure 3: Thin layer chromatography of HWE of Brassica oleracea var. botrytis (A) Presence of polyphenols with an R_f of 0.69 (B) Standard quercetin with an R_f of 0.60.

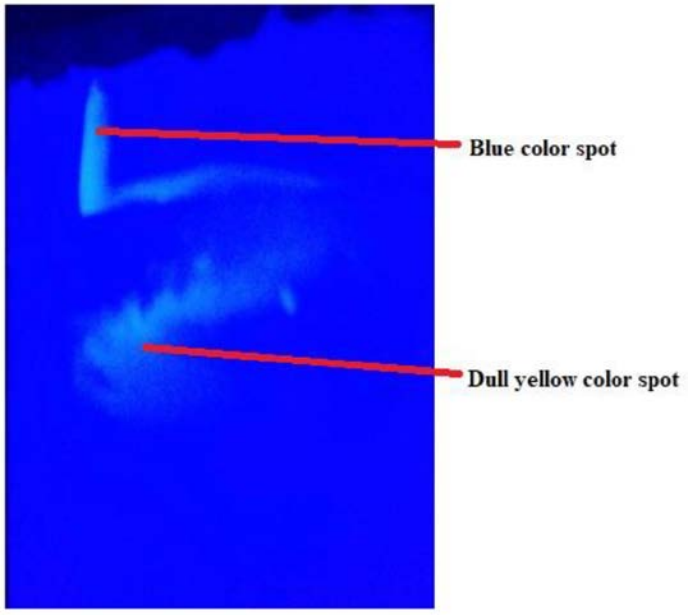

Figure 4: Preparative thin layer chromatography of HWE of Brassica oleracea var. botrytis revealing blue colored and dull yellow-colored spots indicating the presence of polyphenols.

eluate was found to record a near moderate CEase inhibitory activity (28.57 ± 2.12%) which was about one third contribution of polyphenolic content on par compared with HWE based activity. A very scarce reports were available that demonstrated the pancreatic lipase inhibition of *Brassica oleracea var. botrytis* extracts.[37,46] No documentation was available for CEase inhibition of *Brassica oleracea var. botrytis* and our laboratory was first to report the above said property. Likewise, appreciable reports were documented for the presence of polyphenols as flavonoid, phenolic acid, anthocyanins etc., in *Brassica oleracea var. botrytis*, but none had demonstrated the influence of purified polyphenolic fractionate against pancreatic lipase or CEase, and hence, we were again first to describe the CEase inhibitory attribute of *Brassica oleracea var. botrytis.*

CONCLUSION

It was concluded that HWE of *Brassica oleracea var. botrytis* possess significant CEase inhibitory activity. Similarly, a satisfactory inhibition established by the purified polyphenolic eluate of *Brassica oleracea var. botrytis* has showed its significant contribution in the control against various cardiovascular ailments. In future, more studies can be focused to predict the molecular structures of the specific polyphenols that reveal CEase inhibitory property and can be extended towards animal studies to prove the efficacy of purified polyphenols in the regulation of cardiovascular diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CEase: Cholesterol Esterase; **CCD:** Central Composite Design; **RSM:** Response Surface Methodology; **1D TLC:** One Dimensional Thin Layer Chromatography; **2D TLC:** Two Dimensional Thin Layer Chromatography; **CVD:** Cardiovascular Diseases; **LDL:** Low Density Lipoprotein; **HDL:** High Density Lipoprotein; **ACE 1:** Angiotensin Converting Enzyme-1; **OSE:** Organic Solvent Extract; **TPC:** Total Phenolic Content; **pNPB:** para-nitrophenyl butyrate; **HWE:** Hot Water Extract; **PHWE:** Pressurized Hot Water Extract; **MAE:** Microwave Assisted Extraction.

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