Conference Paper

Exploring Neuroprotective Properties of *Centella Asiatica* Extract on Metabolic Change in Chronic Stress-Induced Rats

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Published: 15 March 2021

Publishing services provided by Knowledge E

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Selection and Peer-review under the responsibility of the IVCN Conference Committee.

Abstract

Stress is a mode of adaptive response towards external demands, and prolonged exposure to stress is known to promote aging and neurodegeneration. Several therapies promote neuroprotection but are usually accompanied by adverse consequences. Traditional medicine has been proven as an effective alternative for promoting pharmacological health benefits such as wound healing, boosting memory function and reducing oxidative stress. *Centella asiatica* (CeA) has also gaining attention as an alternative option in promoting neuroprotective activities against neurodegenerative disorders and neuronal injuries. In this study, the neurodegenerative condition of rats was achieved using chronic stress through movement restraint and forced swimming for 21 consecutive days. Here, the neuroprotective properties of three different dosage of CeA (200 mg/kg/day, 400 mg/kg/day and 800 mg/kg/day) was evaluated using metabolomics approach. The administration of CeA shown distinction between untreated group and treated group; and reducing the effect of chronic stress in rats. The extract also demonstrated a significant elevation in several metabolites (lactate, isoleucine, proline, methionine, valine, leucine and glutamine) in rats treated with CeA, particularly in rats administered with 800 mg/kg of CeA. These significant metabolites play important roles in variety of biochemical function of the brain such as the synthesis of protein, energy metabolism, synthesis of neurotransmitter, protection against oxidative stress and compartmentalisation of glutamate. The results of this study may contribute towards greater understanding of molecular mechanism of CeA in promoting neuroprotective properties against neurodegeneration from exposure to chronic stress.

Keywords: *Centella asiatica*, Chronic Stress, NMR-based metabolomics, Serum Metabolites

1. Introduction

Stress can be defined as a mode of adaptive response towards noxious or aversive stimuli [1]. Chronic stress was known as one of the conspicuous causes to aging and neurodegeneration [2]. Brain structures, particularly the limbic region is very susceptible to chronic stress and injuries, and prolonged exposure to chronic stress was markedly associated with severe degradation of the structure [3]. The limbic region is important for cognitive and memory processes, and functional deficits from chronic stress will severely affects cognitive and memory functions [4].

However, there is limited efficient treatment to associated cognitive deficits due to exposure to chronic stress. Therefore, neuroprotection is essential in preserving cognitive and memory functions from exposure to chronic stress. The brain are capable of spontaneous regeneration and reconstutive repair of severed structures, however the rate of neuronal regeneration is usually impeded to the rate of neuronal degradation. Several therapies were notable in promoting neuroprotection properties but is usually accompanied with adverse side effects. In view of adverse side effects of these therapies, there is a need for alternative option that are more efficient and safer to consume. The consumption of traditional herbs was noted as alternative remedy and known for its versatility.

Centella asiatica (CeA) or commonly known as pegaga is creeping plants belong to the Apiaceae family and is native to tropical and subtropical region such as Malaysia and India. [5]. The plant was usually consumed traditionally as fresh salad and the health benefit of CeA consumption has been well documented. Previous reports showed the consumption of CeA helps to promote wound healing of skin, reducing oxidative stress and boosting memory function [6-8]. There has been rising cognizance on the potential neuroprotective properties of CeA. Previous study by Haleagrahara and Ponnusamy showed the positive effects of Centella asiatica (CeA) administration in minimising the effect of neurodegeneration in Parkinsonism animal model [9]. Another study by [10] demonstrated the neuroprotective properties of water CeA extract in rats' hippocampal neurons against Amyloid-b toxicity.

In this study, chronic stress-induced animals were used and administered with different dosages of CeA. Subsequently, 1H NMR metabolomics were used to determine and observe the metabolic changes in the serum samples from chronic stress-induced rats upon the administration of CeA extract. In this study, we sought to assess and further understand any metabolic changes that occur in chronic stress animals upon CeA treatment.

2. Materials and Methods

2.1. Animals

Wistar rats weighing between 200 and 220 g were used in this study. Rats were given free access to certified rodent food (Gold Coin, Malaysia) and water *ad libitum*. Rats were housed with two animals per cage and maintained at 12:12 h dark and light cycle, constant temperature of 25±2°C and relative humidity of 40%. All experimental were performed in accordance to protocols reviewed and approved by Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (ACUC No: UPM/IACUC/AUP-R031/2015).

2.2. Preparation of Extract

Collected CeA leaves were authenticated at Atta-ur-Rahman Institure of Natural Product Discovery (AuRIns) and a sample of CeA plant was deposited (Voucher No: CA-K017). The leaves were washed and dried in hot air oven for three days at 60°C. Dried leaves were ground into course powder using mechanical grinder. 25 g of coarse plant powder were mixed with 250 mL boiling water for 1 h and the mixture was filtered and concentrated to dried powder vacuum at 50°C.

2.3. Experimental Design

Rats were randomly assigned to into five different groups (n=8) and supplemented orally for 21 consecutive days as follows. The negative and positive control groups were supplemented with distilled water. Three groups were supplemented with different dosages of aqueous *Centella asiatica* extract (CAE) respectively; 200 mg/kg/day (CAE 200), 400 mg/kg/day (CAE 400) and 800 mg/kg/day (CAE 800). Rats in respective dosage group were fed using oral gavage with given dosage of CAE daily for 21 days. All rat groups with exception of negative control were left for one hour prior being induced to chronic stress for 30 minutes. Stressor used to induce chronic stress were restrainer and forced swimming. Study was carried out for 21 consecutive days and rats were given free access to food and water. Rats were sacrificed subsequently by decapitation following the last day of treatment.

2.4. 1H NMR Spectroscopy of Serum

Blood samples were collected in vacutainer tube for serum collection during sacrifice. Whole blood was allowed to clot at room temperature for 30 min and centrifuged at 3000 g for 10 min. The resulting serum was transferred stored at -80°C prior to analysis.

The serum samples for 1H NMR analysis were prepared by mixing 200 µL of thawed serum with 400 µL of phosphate buffered saline (PBS; pH 7.4) containing 0.2% TSP. 500 µL of the mixture was transferred into 5 mm NMR tube (Norell Standard Series, Norell Inc., USA). All NMR spectra data was obtained at 26°C on Bruker Avance III 600 MHz NMR Spectrometer (Bruker, USA). The water signal and broad protein resonances were suppressed using combination of PRESAT sequence and Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. The spectral width was 12 KHz with total of 128 scans were obtained into 32K data points and total acquisition time of 480 seconds.

2.5. Data Processing and Multivariate Statistical Analysis

The collected spectra were manually phased and corrected for baseline distortion. The chemical shifts were referenced to TSP as an internal standard. All spectra were carefully aligned using MestReNova software (Version 12.0, Metrelab Research S. L., Spain). The integrals from region of δ 6.0-4.7 ppm were excluded to eliminate the effects of imperfect water suppression in all spectra, and the integrals were normalized to the total integral of the spectrum.

Resulting data matrices were imported into SIMCA-P+ 12.0 software package (Umetrics AB, Sweden) and MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) for chemometrics analysis. Principle components analysis (PCA) was performed to determine the differences in metabolic profiles of all sample groups. Partial least-squares discriminant analysis (PLS-DA) was performed to identify further separation and maximise inter-class variance between sample groups. The heat map for correlation analysis, the hierarchical clustering analysis (HCA) and variable importance in the projection (VIP) between relative level of significant metabolites were determined. Statistical differences of metabolites between groups were performed using Tukey's honest significant difference (Tukey-HSD) multiply comparison and p-values < 0.05 were considered statistically significant.

3. Results

3.1. Identification of metabolites in serum samples

The characteristic ^{1}H NMR signals and its multiplicities were obtained. The identified metabolites were assigned based on tabulated detectable metabolite in animal body fluids by ^{1}H NMR [11-13]. A total of 22 metabolites were observed in the serum sample of NC, PC and treated groups (CAE 200, CAE 400, CAE 800). The identified metabolites were as shown in Table 1.

Table 1: Identified metabolites in treated and untreated group.

Number	Metabolites	Chemical Shifts (±0.025 ppm)
1	Leucine	0.94; 0.95; 1.67; 1.70; 1.73; 3.72
2	Valine	0.98; 1.03; 2.26; 3.60
3	Isoleucine	0.93; 1.00; 1.25; 1.46; 1.97; 3.66
4	Threonine	1.32; 3.58; 4.25
5	Lactate	1.32; 4.11
6	Alanine	1.47; 3.78
7	Lysine	1.43; 1.50; 1.72; 1.88; 1.91; 3.02; 3.75
8	Proline	1.98; 2.02; 2.34; 3.33; 3.41; 4.12
9	Methionine	2.11; 2.13; 2.19; 2.63; 3.85
10	Glutamine	2.11; 2.14; 2.43; 2.46; 3.77; 6.87; 7.59
11	Citrate	2.53; 2.69
12	Creatine	3.03; 3.92
13	P-creatine	3.03; 3.94
14	Creatinine	3.03; 4.05
15	Phenylalanine	3.11; 3.27; 3.99; 7.32; 7.37; 7.42
16	Betaine	3.25; 3.89
17	Taurine	3.25; 3.42
18	Glucose	3.24; 3.39; 3.40; 3.46; 3.48; 3.53; 3.70; 3.72; 3.76; 3.82; 3.84; 3.89; 4.64; 5.23
19	Glycine	3.55
20	Glycerol	3.55; 3.65; 3.78
21	Fructose	3.55; 3.56; 3.59; 3.67; 3.70; 3.70; 3.79; 3.79; 3.82; 3,89; 3.99; 4.01; 4.10; 4.11
22	Serine	3.84; 3.94; 3.98

3.2. Metabolite analysis of serum samples

An unsupervised PCA model was fitted for binned spectra from serum samples collected from NC, PC and treated groups (CAE 200, CAE 400, CAE 800). The first principal component and second principal component accounted for 92.5% the total variations. Therefore, the scores plot and loading plot showed good summaries of the data set. The

DOI 10.18502/kls.v6i1.8771

data was auto-fitted and PCA analysis resulted variation between different treatment groups. The PCA plot showed differential tendency of metabolic profiles between groups during administration of CAE. The PC group was distinctly separated in the first principal component with PC being in negative t1 region as shown in Figure 1. Group NC was observed in positive t1 region. A separation was observed between treated groups with group CAE 200 being in negative t1 region; group CAE 400 being in between of positive and negative t1 region; and CAE 800 being in positive t1 region.

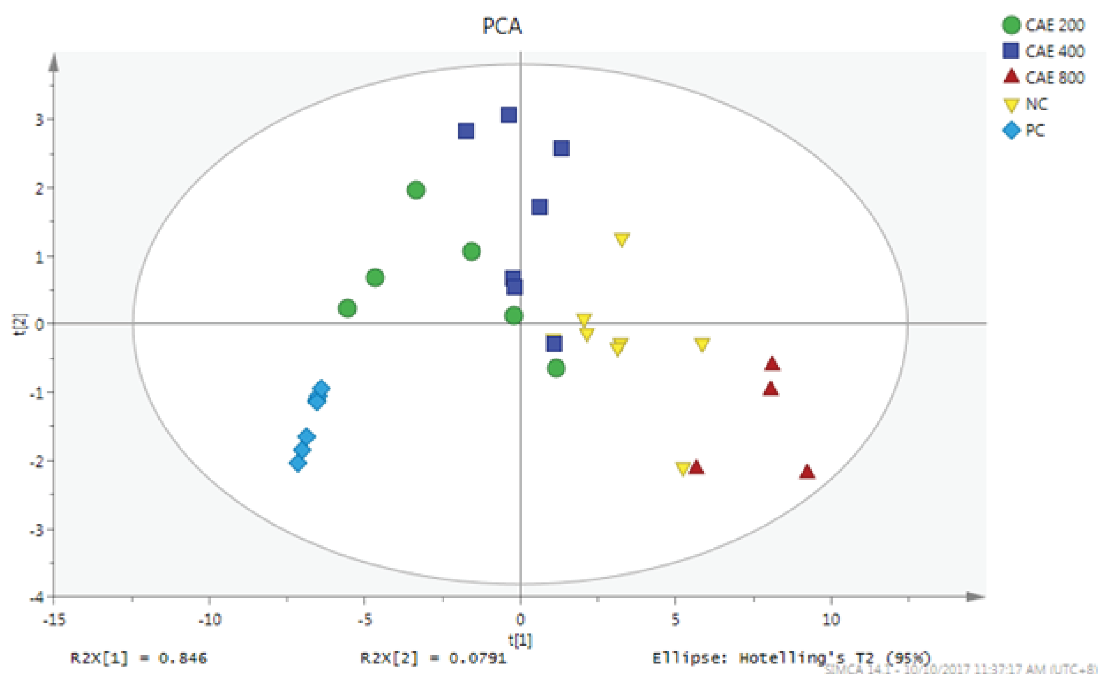

Figure 1: The PCA scores plot. NC=negative control, PC=positive control, CAE=*Centella asiatica* extract, 200 = 200 mg/kg/day, 400 = 400 mg/kg/day, 800 = 800 mg/kg/day.

A supervised PLS-DA was constructed to identify the metabolites that were significantly associated with chronic stress and treatment of CAE. The PLS-DA score plot is as shown in Figure 2. The first principal component 1 and principal component 2 were described with total variance of 92.0% with class discriminant R2Y values of 0.840 and Q2 values of 0.627 respectively. The observation indicates the suggested metabolites were significantly affected by administration of CAE. The PC group is clustered to negative t1 region of the score plot. Different clusters were observed in the treated group CAE 800) and NC, which clustered to positive t1 region of the score plot. The observation exhibited a clear separation between treated and untreated chronic stress group. Treated group CAE 200 however clustered in negative t1 region of the score plot but showed a slight separation from the PC group towards NC group. Treated group CAE 400 was clustered in the middle of the score plot, nearing towards NC group.

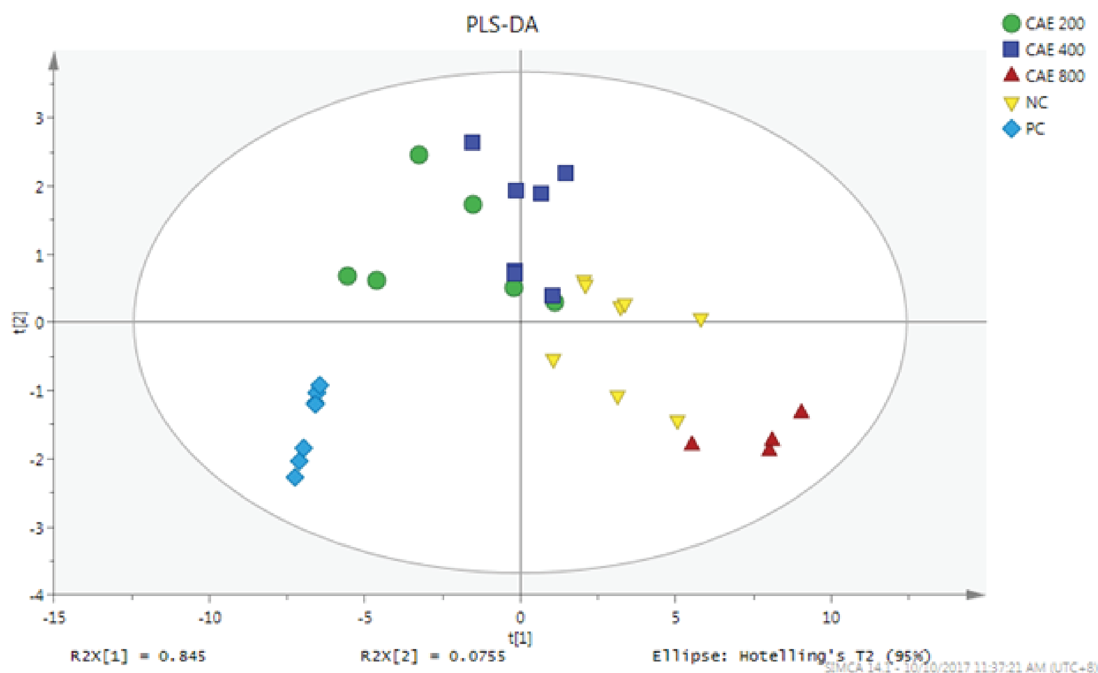

Figure 2: The PLS-DA scores plot. NC=negative control, PC=positive control, CAE=*Centella asiatica* extract, 200 = 200 mg/kg/day, 400 = 400 mg/kg/day, 800 = 800 mg/kg/day.

Heat map analysis was constructed based on the results obtained from PLS-DA and variance importance in projection (VIP) plot was constructed to identify changes in metabolites in treated and untreated group. The metabolomics pattern of key metabolites was expressed as coloured square in heat map, by which the colour based scaled from -3 as the lowest and 3 as the highest. The heat map constructed was as shown in Figure 3. The treated group exhibited higher levels of observed metabolites. These analyses were consistent with separation of treated and untreated groups based on the PLS-DA score plot. The most significant contributing variables was identified using VIP analysis of PLS-DA model as shown in Figure 4. A total of 7 metabolites was identified due to their VIP values of more than 0.5, which indicates as significant metabolites in the separation.

4. Discussion

In this study, we looked at the metabolic changes due to the effects of administration of CeA in chronic stress-induced rats. Metabolic analysis identified 12 metabolites whose profile significantly affected the distinction between treated and untreated group after the administration of CeA. The heatmap and VIP scores of the 12 significant metabolites showed a separation trend in the PCA and PLS-DA analysis, as well as the biomarkers of treatment with CeA extract.

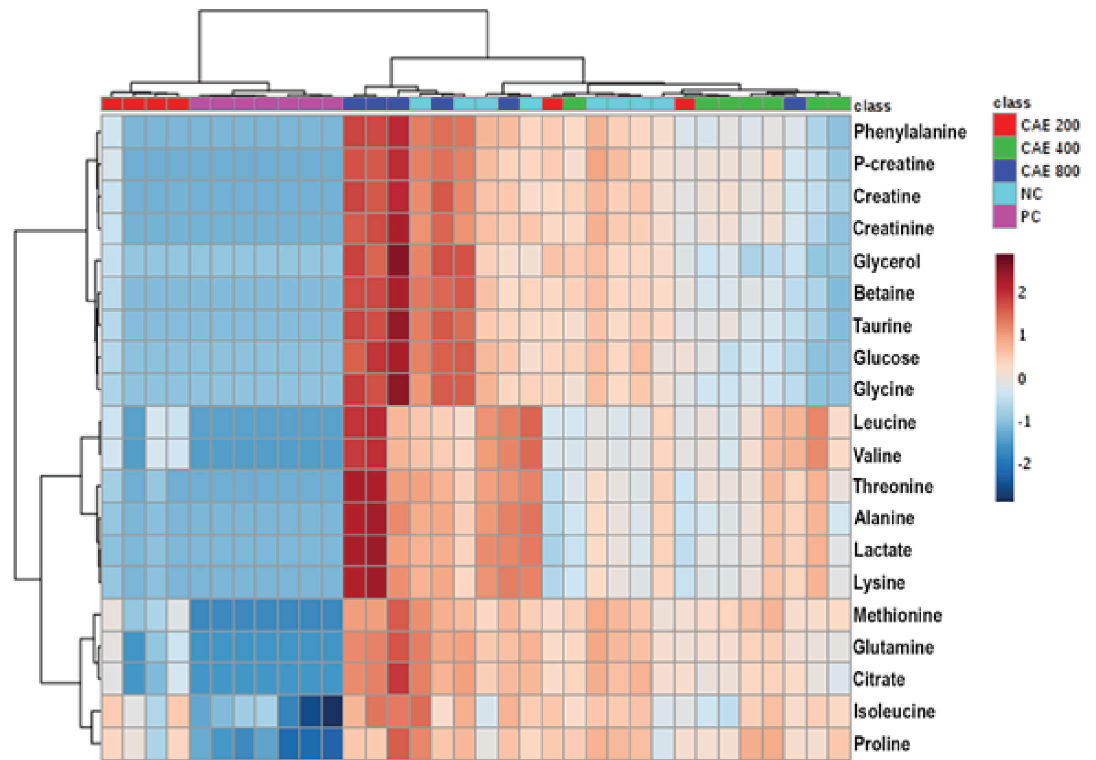

Figure 3: The heatmap analysis. NC=negative control, PC=positive control, CAE=*Centella asiatica* extract, 200 = 200 mg/kg/day, 400 = 400 mg/kg/day, 800 = 800 mg/kg/day.

In the present study, significant changes were observed in lactate in correlation with the highest VIP scores. Lactate was observed to be significantly higher in treated group compared to PC group. The lactate is mainly associated with energy metabolism via the tricarboxylic acid (TCA) cycle [14]. Previous studies described a model by which glucose from vasculature was metabolised by glial cells into lactate and later transported to neurons for TCA cycle [15]. Elevated level of lactate was shown to be associated with stimulated neuronal activities, indicating lactate as source of energy for neurons [16]. Therefore, higher lactate level in treated group indicated higher energy metabolism by which may lead to stimulated neuronal activities.

Isoleucine, leucine and valine were also identified as markers, which is significantly higher in treated group compared to PC group. The three branched-chained amino acids (BCAAs) were detected the highest in CAE 800 group and lowest in the PC group. BCAAs play an important role in variety of biochemical functions of the brain; which includes synthesis of protein, energy metabolism, compartmentalisation of glutamate and synthesis of neurotransmitters [17]. Elevated levels of isoleucine, leucine and valine indicated the lower level of amine transmitters, known as 5-hydroxytryptamine (5-HT). BCAAs can compete with its precursor, tryptophan, which helps with the transport across

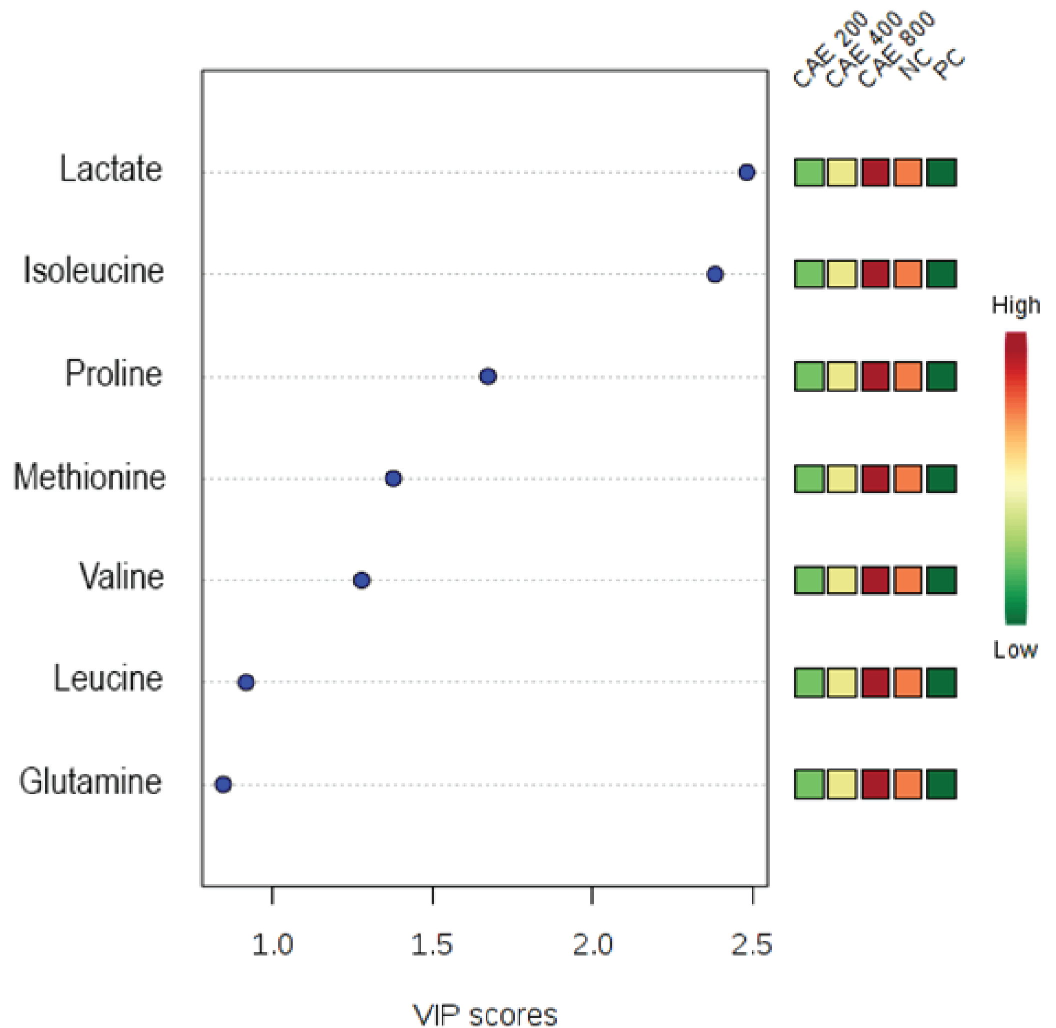

Figure 4: VIP values derived from PLS-DA. NC=negative control, PC=positive control, CAE=*Centella asiatica* extract, 200 = 200 mg/kg/day, 400 = 400 mg/kg/day, 800 = 800 mg/kg/day.

the blood-brain barrier [18]. Elevation of BCAAs will reduce the intake of tryptophan by the brain and thus, indicated the reduction of central fatigue [19].

Glutamine was identified to be significantly higher in treated group compared to PC group. Glutamine performed an important role in neurotransmitter synthesis. Glutamate were one of the important major neurotransmitters at excitatory synapses and mediated by glutamine-glumatate cycles [20, 21]. Glutamate and glutamine are enzymatically interconvertible and the cycle is energy dependent [22]. Glutamate were also performed an important role in synaptogenesis during brain development [23]. Elevation of glutamine may indicate higher activity of neutransmitter synthesis and synaptogenesis in the brain.

Proline was identified as one of the metabolites with significant changes. Proline was observed to be significantly higher in treated group compared to PC group. Proline

was shown to possess protective effect against oxidative stress and apoptosis [24]. Previous study by Li et al. showed the effects of metabolites such as proline, glutamine and alanine in minimising the effect of reactive oxygen species (ROS) and hydroxyl radical-induced apoptosis by supressing the release of cythochrome c and activation of caspace-3, caspace-8 and caspace-9 [25]. Elevation of proline may indicate reduction of oxidative stress in rats.

Methionine was observed as significant metabolites that cause the distinction between treated group and untreated group. Methionine is an essential amino acid that is important for normal growth and development in mammals [26]. Methionine is an important metabolite for protein synthesis and the methylation cycle by which it will be converted to S-adenosylmethionine (SAM) [27]. Elevation of methionine may indicate there is higher activities of growth and development in rats.

5. Conclusion

In summary, distinction between treated group and untreated group was observed in metabolic patterns in the blood serum. The changes in metabolic pattern was related to amino acid metabolism and energy metabolism. The distinctly expressed metabolites between CeA treated rats and untreated rats indicated that the metabolic pathway involving amino acid metabolism and energy metabolism were affected by the extract. Metabolic disparity between treated and untreated group suggested that the administration of CeA is capable to reduce the effect of chronic stress in rats and collectively providing insights on the effect on CeA in chronic stress model.

Funding

The authors are gratefully acknowledged the support given by Ministry of Agriculture (MoA), Malaysia (Project Number: 100-MOA/RMI/16/6/2); as well as Universiti Teknologi MARA for Geran Inisiatif Penyeliaan (GIP) (Project Number:600-IRMI/MyRA 5/3/GIP) and Geran Penyelidikan LESTARI (Project Number: 600-IRMI/MyRA 5/3/LESTARI).

Acknowledgement

The authors are also thankful to Universiti Teknologi MARA (UiTM) for the support and facilities used during the commencement of this study.

Conflict of Interest

The authors have no conflict of interest to declare.

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