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Moringa oleifera, *Cyclea barbata*, *Centella asiatica* Leaves Extract Intervention Ability to Improve *Fetus* Development and Blood Protein Levels in Malnutrition Pregnant Model Mice

Intervensi Ekstrak Daun Moringa oleifera, Cyclea barbata, Centella asiatica untuk Meningkatkan Perkembangan Fetus dan Kadar Protein Darah pada Model Mencit Bunting dengan Malnutrisi

Juliana Christyaningsih[1]*, Nur Hatijah[1], Husnul Halimah[1]

[1]Politeknik Kesehatan Kemenkes Surabaya, Indonesia

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****Correspondent:***
Juliana Christyaningsih
juliana.christy123@gmail.com

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ABSTRACT

Background: Pregnant women experiencing chronic energy deficiency are at risk of giving birth to low birth weight and premature (stunted) babies.
Objectives: In an effort to prevent stunting, the study investigates the effects of a 1:1:1 ratio of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaf extract on fetal growth and blood protein levels.
Methods: This research used a laboratory experimental approach with 25 adult female Mus musculus Balb/C mice as a model of malnourished pregnancy, was divided into 5 groups: pre-test, positive control, negative control, placebo and test group. *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaf extracts are administered to the test group and positive control in 1:1:1 ratio.
Results: The study results indicated that in pregnant mice without malnutrition receiving a mixture of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaf extracts, the average fetal body length was 9.7% higher and their weight was 7.4% heavier than pregnant mice with experiencing chronic energy deficiency.
Conclusions: The administration of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaf extract can contribute to improving fetal development and blood protein levels. However, its effectiveness is more pronounced in normal pregnancies than in pregnancies with chronic energy deficiency as it can prevent stunting.

INTRODUCTION

Pregnant women facing low energy and protein intake are susceptible to developing chronic energy deficiency conditions. A risk factor of this condition is an upper arm circumference <23.5 cm, which leads to the birth of low-weight and premature babies[1]. The repercussions of birthing low low-weight baby include increased security risks, as well as impaired growth and development of the child. Furthermore, chronic energy deficiency can indirectly contribute to maternal death. Insufficient consumption of essential macronutrients and micronutrients during pregnancy may lead to nutritional issues such as anemia and persistent protein-energy deficits. This elevates the likelihood of delivering babies with low birth weight, as well as increasing maternal and infant mortality[2].

Stunting not only results in reduced height but also hinders children from reaching their full growth potential. Early deficiencies in diet can lead to increased mortality rates among infants and children due to illnesses and long-term posture issues in adulthood. Furthermore, decreased cognitive abilities associated with stunting result in long-term economic losses for Indonesia[3]. The initial 1000 days of life represent a crucial period, signifying the onset of stunting cases with enduring consequences extending to the subsequent "1000 Days of Nutrition." Educate the public about the importance of the first 1000 days of a child's life in implementing nutrition to ensure optimal growth and development[4].

Research on *Moringa oleifera* highlights the health advantages of its leaves, pods, seeds, and flowers. Safaringga's study revealed that *Moringa oleifera* leaf extract can significantly boos breast milk production, as evidenced by a p-value 0,000<0,05[5]. *Moringa oleifera* leaves demonstrate the potential to enhance breast milk production, as indicated by improvements in infant weight, urination frequency, fecal expulsion frequency,

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and breastfeeding[6]. Tri Suhartini's, 2018 study revealed that biscuits containing *Moringa oleifera* leaves had a significantly higher protein and calcium contents, with increases of 18.2% and 78,97%[20]. The leaves contain essential amino acids ensuring the suitability for consumption by children with imperfect digestion to meet protein needs. The body has numerous roles for essential amino acids, which influence various organ and cell functions[7].

Cyclea barbata plants thrive in lowland areas situated approximately 800 meters above sea level. Phytochemical analysis of the leaves, conducted using maceration and infusion methods, revealed the presence of flavonoid, alkaloid, saponin, and tannin. In Mahadi's study on phagocytic activity, *Cyclea barbata* leaf extract in ethyl acetate exhibited the highest phagocytic properties, outperforming chloroform and ethanol extracts based on thin-layer chromatography results. The ethyl acetate extract demonstrated potent immunomodulatory and antioxidant properties due to rich content of phenolic, flavonoids, tannins, and terpenoids[8]. Antioxidants play a crucial role in protecting body cells from oxidative stress and free radicals. Ensuring sufficient intake of specific antioxidants like vitamin C, vitamin E, and selenium during pregnancy promotes healthy fetal development by lowering the chances of oxidative stress[9,10]. Furthermore, protein is an essential nutrient required for supporting fetus growth and tissue development during pregnancy[11,12]. Certain antioxidants, such as vitamin C and E, can impact protein metabolism within the body by facilitating absorption and collagen formation, essential for maintaining tissue structure.

Centella asiatica (L) contains a range of active compounds such as saponins, alkaloids, flavonoids, tannins, triterpenoids and steroids, among others. These substances include antioxidants that offer various health benefits to humans. Furthermore, *Centella asiatica* (L) contains minerals such as magnesium, calcium. potassium, sodium. iron, phosphorus, along with pectin, amino acids, essential oils, and B vitamins. The application of fresh *Centella asiatica* leaves and its decoction has been shown to enhance the memory of experimental animals, particularly mice with brain cell necrosis. Expansive study efforts have led to the development of *Centella asiatica* into a supplement and functional food product. These items encompass jelly candies and lozenges containing *Centella asiatica* extract. The jelly candies are infused with *Centella asiatica* extract to enhance children's memory, while the lozenges utilize *Centella asiatica* extract with mannitol solvent as a memory-enhancing agent. Administration of its ethanol extract has shown efficacy in enhancing bone calcification and preventing stunting in *zebrafish,* yielding a 99.60% increase in body length from pre- to post-hatching stages. *Centella asiatica* extract containing 70% of a type of acid (*asiatic acid, madecassic acid, asiatoside*) has therapeutic potential in addressing skin problems, blood flow issues, and swollen veins. It helps the connective tissue work better through the augmentation of glycosaminoglycan that fills the space between the collagen fibers, thereby promoting tissue health. The plant extract also protects the heart by reducing inflammation, cell death, and oxidative stress. *Asiatic acid*, a key compound, has shown healing properties and efficacy in vivo by modulating molecular pathways in mice. It also reduces the amount of ROS in heart cells exposed to hydrogen peroxide in vitro. The struggle to reduce stunting rates in Indonesia must begin by improving the nutritional status of young girls, pregnant women and fetuses during period "1000 days" of their lives. Integrating *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaves as alternative measures can significantly contribute to addressing the stunting issue. This study aims to investigate the impact of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaf extracts on protein levels in the blood and fetal development, aiming to prevent stunting.

METHODS

An experimental study that was conducted in the laboratory used adult female *Mus musculus* strain Balb/C mice as models for pregnant animals experiencing malnutrition. The Health Research Ethics Commission of the Ministry of Health Surabaya Polytechnic received approval for the use of these experimental animals under certificate number EA / 829 / KEPK-Poltekkes_Sby / V / 2022. This study followed the principles outlined in the Helsinki Declaration, as confirmed by the Health Research Ethics Commission of the Surabaya Ministry of Health (Protocol №5, 26.05.2016). Three ratios are utilized to formulate the food products from *Moringa oleifera, Cyclea barbata*, and *Centella asiatica*, namely Formula 1, 2, and 3, at 1:1:1, 1:1:2, and 1:2:1, respectively. After sensory testing to evaluate texture, taste, color, and flavor, Formula 1 had the most preferred results. The nutrient composition of the blend was analyzed at the Faculty of Pharmacy Laboratory, Universitas Airlangga Surabaya, using 1:1:1 ratio of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaves. The animal model adopted in this study was *Mus musculus (mice)* Balb / C strain, with a body weight ranging from 25-30 grams in adult females aged 10 weeks. Following that, randomly selected mice from the Faculty of Veterinary Medicine at Airlangga University were induced into a model of malnourished pregnancy. These mice were housed in an experimental animal cage at Airlangga University's Faculty of Medicine, Department of Biochemistry.

Procedure

Adult female *Mus musculus* Balb/C mice, 10 weeks old and weighing between 25 to 38 grams, were used as a malnutrition model in this study. The mice exhibited signs of good physical health such as clear eyes, shiny fur, and active movement. Mice were malnutrition by feeding with a low-protein diet, which led to gradual weight loss. To induce pregnancy, samples in the known estrus period were selected based on signs of vulvar swelling. The estrous period lasted for four to five days, with a mating window of twelve to fourteen hours. Female mice in estrus were housed in cages with a male at a ratio of 3:1, and the detection of a vaginal pug marked the beginning of pregnancy as day 0. Mice usually have a gestation period that spans from 19 to 21 days, and they typically have an average litter size of 6 to 15 pups. Surgical procedures were performed on either day 21 of pregnancy or during delivery while under

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anesthesia.

In this study, the 25 mice were provided with pellet starters and had ad libitum access to water during the adaptation phase. They were housed in a maintenance room with an optimal temperature maintained at 22±3ºC, a relative humidity range of 30 - 70%, and a lighting cycle of 12 hours of light followed by 12 hours of darkness. During the study, the health condition of the experimental animal was closely monitored, including assessments of body weight, fur condition, eye clarity, presence of nasal mucus, incidence of diarrhea, and motor activity. The animals underwent approximately one week of acclimatization to the cage environment before being grouped into five treatment groups, each containing four mice, and were fed for three weeks with ad libitum access to drinking water. Malnutrition was induced through a three-week regimen of low-protein food, coinciding with the start of the pregnancy process.

There are five groups in the study: (1) Pretest group, mice with chronic energy deficiency; (2) Placebo group, normal pregnant mice receiving no treatment; (3) Positive Control group, normal pregnant mice with chronic energy deficiency condition receiving no treatment; (4) Normal pregnant mice administered with formulations of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaf extract; (5) Treatment group, mice with chronic energy deficiency who received daily sonde administration of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* in sodium carboxymethyl cellulose (NaCMC) solution. During the treatment phase, the mice had access to food and water without restrictions. The parameters assessed in this research encompass fetal weight and length, along with the maternal blood protein content. This is significant because maternal malnutrition can influence both maternal protein levels and the nutritional condition of the fetus. Fetus body weight and length are measured using a balance and ruler, respectively, while maternal blood protein is measured using the Biuret method.

RESULTS AND DISCUSSIONS

Table 1 showed the nutrient content in a mixture of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaves at 1:1:1 ratio was analyzed at the Laboratory of the Faculty of Pharmacy, Universitas Airlangga:

Table 1. The research examined the nutritional composition of food items containing *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaves in a 1:1:1 proportion.

Nutrient Content of Food Products	Inspection Method	Rate
Carbohydrate	SNI 01-2891-1992	2.9% ± 0.005
Total Fat	SNI 2715:2013	0.2% + 0.026
Protein	SNI 2715:2013	0.81% + 0.048
Fiber	SNI 01.2891-1992	0.88% + 0.026
Calcium	AAS	0.032% + 0.097
Magnesium	AAS	0.002 % + 0.006
Potassium	AAS	0.39 % + 0.022
Copper	AAS	0.028 ppm
Iron	AAS	23.9 ppm + 0.008
Phosphor	Spectrophotometry	121.2 ppm + 0.053
Vitamin B1	HPLC	Positive
Vitamin B6	HPLC	Positive
Vitamin E	HPLC	Positive
Polyphenols	Qualitative	Positive

The study investigated the impact of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaf extract using 1:1:1 formula on pregnant mice. The extracts were orally administered via a sonde, and measurements were taken on the body weight and length of the fetuses in grams and centimeters, respectively. Additionally, blood sample were collected from the mother mice's hearts to assess protein levels. The table presents the results from the analysis:

Table 2. The data on blood protein levels, the number of brooding fetuses, and the body weight and body length of fetuses in the 5 groups

Group	Code	Mice Blood Protein Levels (g/dL)	Number of Brooding Fetuses	Fetus Weight Average (g)	Fetus Body Length Average (cm)
Placebo (Normal Pregnant Mice)	PL1	7.8	10	2.0	3.07
	PL2	7.2	8	2.0	3.03
	PL3	7.2	5	2.0	3.22
	PL4	8.4	9	2.11	3.13
	PL5	7.7	8	2.03	3.11
Average		7.66	8	2.028	3.112
The Negative Control Group consisted of normal pregnant mice that received *Cyclea Barbata*,	KN1	7.6	8	2.22	3.69
	KN2	8.1	3	2.00	2.90
	KN3	7.8	12	2.00	3.00

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Group	Code	Mice Blood Protein Levels (g/dL)	Number of Brooding Fetuses	Fetus Weight Average (g)	Fetus Body Length Average (cm)
Centella Asiatica and *Moringa Oleifera* leaf extracts	KN4	7.7	9	2.32	3.53
	KN5	7.6	9	2.34	3.42
Average		7.76	8.2	2.176	3.308
Pre-Test (Chronic Energi Deficient Mice)	Pre 1	6.0			
	Pre 2	5.9			
	Pre 3	6.0			
	Pre 4	6.2			
	Pre 5	5.4			
Average		5.9			
Positive Control (Pregnant Mice with Chronic Energi Deficiency)	K.Pos 1	5.9	8	2.13	3.15
	K.Pos 2	5.3	7	2.00	3.19
	K.Pos 3	5.3	4	2.00	3.00
	K.Pos 4	6.1	2	2.00	3.10
	K.Pos 5	5.5	2	2.00	3.00
Average		5.62	4.6	2.026	3.088
Treatment (Pregnant Mice with Chronic Energi Deficiency + *Cyclea Barbata*, *Centella Asiatica,* and Moringa Oleifera Extracts)	KP1	5.7	8	2.00	3.15
	KP2	5.4	6	1.92	2.90
	KP3	6.1	5	2.00	2.80
	KP4	7.8	6	1.92	3.08
	KP5	7.1	6	2.00	2.83
Average		6.42	6.2	1.968	2.952

PL = Placebo, KN = Negative Control, Pre = Pre-test, K.Pos = Positive Control, KP = Treatment

Based on the study results, that pregnant mice without malnutrition who were administered a mixture of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaf extract showed fetuses that exhibited an average increase of 9.7% in length and 7.4% in weight compare to the control group. When compared to the placebo group pregnant mice under normal conditions, the condition of fetuses in the treatment group was lower. This indicates that the use of the mixed formula was less effective in preventing stunting. Maternal protein malnutrition has profound effects on the liver health of FGR offspring, affecting metabolic pathways and potentially predisposing them to liver disease later in life. The research findings indicated that the mean blood protein concentrations were reduced in the pregnant mice group suffering from chronic energy deficiency when compared to the malnourished group receiving a combination of the extracts. However, these protein levels remained lower than those of the adequately nourished pregnant mice group. This suggests that chronic energy deficiency conditions can hinder liver function, resulting in inadequate protein production and indirectly impacting the fetus through the condition of the mother mice. The results of the negative control group were the most favorable due to the presence of micronutrient, phytochemical, and antioxidant in the combination of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaves. These nutrients are essential for preventing stunting.

The study by Anggraini demonstrated that chronic energy deficiency during pregnancy can affect blood protein levels. The research showed a notable difference in maternal serum protein levels between chronic energy deficiency and normal conditions[19]. Low blood protein levels in pregnant women can indirectly affect fetal development in the womb. Chronic energy deficiency conditions, observed in both the positive control and treatment groups, can prolong the pregnancy process due to compromised performance of pregnancy-supporting steroid hormones caused by low production of hormone-binding proteins. The impact on blood protein levels due to chronic energy deficiency during pregnancy can indirectly influence fetal body weight and length. The positive control group in the study displayed decreased numbers of fetuses, reduced body weight and height compared to other groups, resembling stunting, a type of malnutrition marked by below-average height for age.

Moringa oleifera, Cyclea barbata, and *Centella asiatica* are widely available plants in Indonesia, offering economic value and beneficial nutritional content. The presence of protein and micronutrients is essential for bodily functions. Therefore, a deficiency in micronutrients can lead to the downregulation of amino acids in the body[13]. Proteins serve various roles in the body, such as acting as buffers to maintain pH balance and functioning as antibodies in the immune system to defend against infections and diseases[14]. According to a study conducted in Uganda, *Moringa oleifera* plants have been utilized in treating 24 chronic metabolic diseases, including stunting[15,16]. In general, the most commonly used part of the *Moringa oleifera* plant is the leaf due to its high content of carotenoids, saponins, phenolic acids, glucosinolates, alkaloids, polyphenols, flavonoids, isothiocyanates and tannins[17]. Different compounds found in herbs can influence inflammatory and genomic processes, including proteomics, metabolomics, and transcriptomics, which are particularly relevant in cases of stunting[18]. It's crucial to ensure sufficient nutrient intake during pregnancy to avoid disturbances in genomic processes and inflammation, both of which can lead to stunting.

Stunting is attributed to alterations in cell DNA, with nutrigenomic and epigenetic properties of nutrients

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capable of inducing genetic disruptions. Unlike epigenetics, which examines DNA variations in chromosomes and alterations in gene expression without modifying the DNA sequence, nutrigenomics focuses on the impact of foods on gene expression. Epigenetic modification such as methylation, histone deacetylation, microRNA (miRNA) regulation and maintenance of chromosomal stability do not alter the nucleotide sequences of DNA strands and histones. Nutrients, despite not directly changing DNA and histones, influence processes like protein transcription, DNA repair, replication, and chromatin structure. They can modify metabolism by changing the concentration of intermediates or substrates, and they can influence gene expression by acting as ligands for transcription factor receptors. Additionally, nutrients can influence various biological activities by signaling via transduction routes.

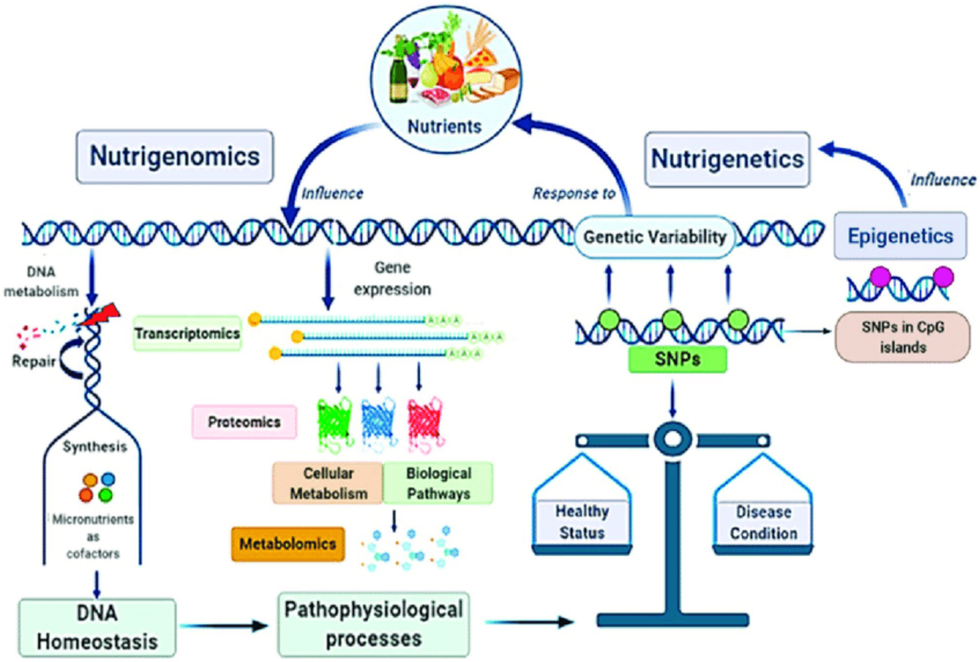

Figure 1. Nutrigenomics and nutrigenetics play crucial roles in understanding metabolic dysfunction associated with fatty liver disease, offering novel insights and future perspectives [19]

The mixture of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaves includes vegetable proteins that function as ligands in the DNA sequence promoter, assisting in the transcription process and thereby promoting protein synthesis[20]. The synthesized protein, essential for combating stunting, is a result of gene expression [21]. Additionally, vegetable fatty acids found in leaf formulations influence the digestion process. Specifically, *alpha-linolenic acid* (PUFA) has the capability to generate *Docosa Hexaenoic Acid* (DHA) or *Eicosa Pentaenoic Acid* (EPA) through de novo synthesis[22]. Upon stimulation by these fatty acids, PPAR-γ relocates to the nucleus and enhances the production of adiponectin. Furthermore, *Docosa Hexaenoic Acid* (DHA) or *Eicosa Pentaenoic Acid* (EPA) activate *Adenosine Monophosphate Protein Kinase* (AMPK), thereby improving beta-oxidation and reducing lipid accumulation.

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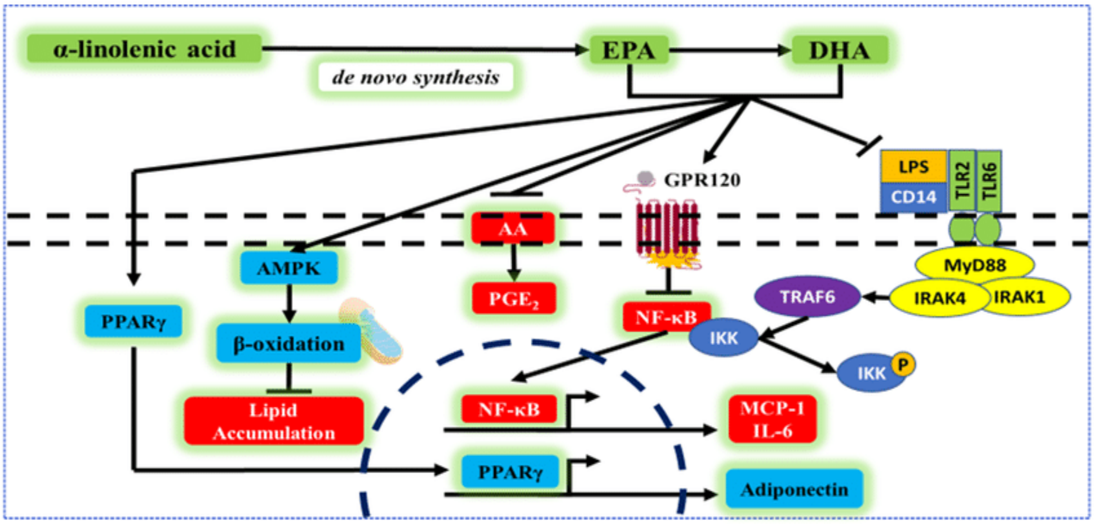

Figure 2. Mechanism. of α-linolenic acid in Nutrigenomics (Modified From Rodríguez-Cruz [22])

The minerals and vitamins present in *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaf formulations contribute to nutrigenomics by enhancing enzyme performance as enzyme cofactors, thus influencing protein function. The leaf mixture, containing vitamins B6 and B12, can be converted into methionine, which is then transformed by the enzyme *DNA methyltransferase* (DNAMTase) into *S-adenosylmethionine* (SAM). Subsequently, SAM enters the cell nucleus to execute epigenetic modifications crucial for protein synthesis and nutrigenomic activities[20]. The study findings indicate that the treatment groups and the negative control group, consisting of normally pregnant mice administered with leaf extracts from *Moringa oleifera, Cyclea barbata*, and *Centella asiatica*, demonstrated better outcomes when compared to positive control group. Pregnant mice with chronic energy deficiency are included in positive control group.

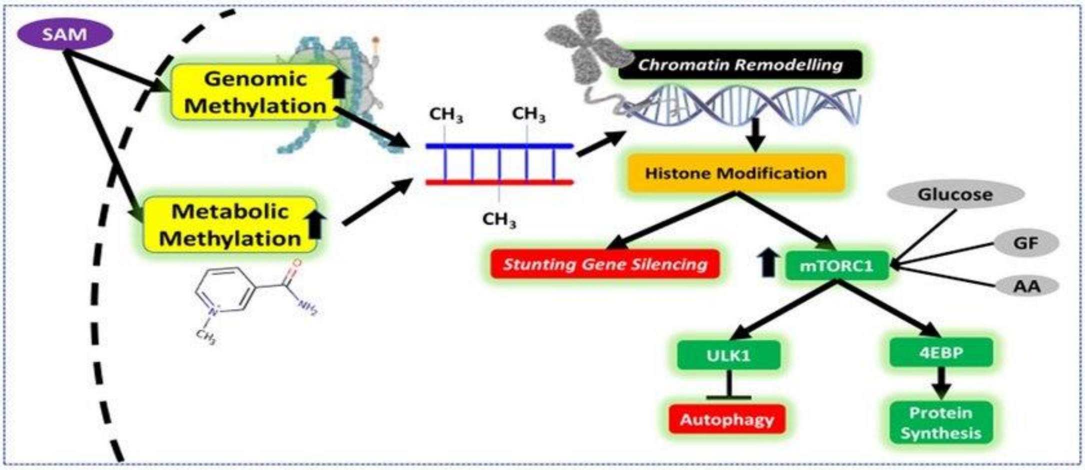

Figure 3. Mechanism of Action of SAM in Nutrigenomics (Modified From Huang [20])

The comprehensive processes explained earlier underscore the substantial impact of nutrigenomics derived from *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaves in averting stunting, primarily attributable to their rich composition of micronutrients.

CONCLUSIONS

The conclusion of this research is that administration of *Moringa oleifera, Cyclea barbata*, and *Centella asiatica* leaf extracts has a beneficial impact on blood protein levels and fetal development. However, this effect was diminished when given to pregnant mice with chronic energy deficiency. Conversely, it was more effective in preventing stunting when administered during normal pregnancy.

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CONFLICT OF INTEREST AND FUNDING DISCLOSURE

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AUTHOR CONTRIBUTIONS

JC: conceptualization, investigation, methodology, supervision, writing–review and editing; NH: formal analysis, resources; HH: writing–original draft, writing–review and editing. All authors participated in contributing to text and the content of the manuscript, including revisions and edits. All authors approve of the content of the manuscript and agree to be held accountable for the work.

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