ANTI-OBESITY AND ANTI-OXIDATIVE EFFECTS OF METHANOLIC 
Albizia myriophylla (L. BENTH) BARK EXTRACT IN OBESE MICE

AZMAH SA’AT

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By
AZMAH SA’AT

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

July 2017
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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AZMAH SA’AT

July 2017

Chairman : Goh Yong Meng, DVM, PhD
Faculty : Veterinary Medicine

Globally and in Malaysia, obesity causes early morbidity and mortality and is costly to treat. The antioxidant activities in herbal plants have been suggested as one of the mechanisms working against obesity. Hence, this study examined the antioxidant activity of Albizia myriophylla bark or known locally as tebu gajah. Its effect on anti-obesity via in vivo test in high-fat diet-induced obesity in mice consisting of the normal control group (NC), the high-fat diet control group (HFDC), the high-fat diet group treated with Albizia myriophylla methanol extract at 10 mg/kg, 20 mg/kg and 30 mg/kg for 5 weeks. Antioxidant activity of the methanol extract and its derived fractions namely hexane, chloroform, ethyl acetate, butanol and a residual aqueous fraction of the bark of ABZ was assessed. In (1,1-diphenyl-2-picrylhidrazyl) radical scavenging test, (2-2′-azinobis 3-ethyl-6-sulfonic acid) radical scavenging test and reducing activity on ferrous iron test, the total antioxidant capacity was found to be varied in different fractions. The IC$_{50}$ calculated value of the three assays showed that the methanolic extract of ABZ bark had the lowest IC$_{50}$ value for each assay, compared to the other extracts signifying highest antioxidant activity and was selected for use in the anti-obesity study. The body weight of mice, adipose cellularity study, and levels of cholesterol, triglyceride, low-density lipoprotein (LDL), the malondialdehyde levels in the muscles of the obese mice, the serum aspartate transaminase (AST) and serum alanine transaminase (ALT) were analysed. Results showed significant weight loss with significant reduction in the number and size of adipose cells in the mice treated with 20 mg/kg and 30 mg/kg of methanolic extract of ABZ bark. In addition, there was a significant reduction of blood cholesterol, triglyceride and LDL following treatment with 20 mg/kg and 30 mg/kg of methanolic extract of ABZ bark, compared to HFDC. Furthermore, the significant reduction of AST, ALT and malondialdehyde in the 20 mg/kg and 30 mg/kg treated group, compared with HFDC, suggest the safety of methanolic extract of ABZ bark in a mice model. The regulatory effects of methanolic extract of ABZ bark on genes including hormone sensitive lipase (HSL), peroxisome proliferator-activated receptors α and γ (PPAR α and PPAR γ), sterol regulatory element-binding protein-2 (SREBP2) and Stearoyl-Coenzyme A-Desaturase (SCD)
gene expression and adipocytokines including tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), adiponectin and leptin involved in lipid metabolism were also studied. The obese mice treated with the 30 mg/kg of methanolic extract of ABZ showed significant up-regulated of PPARα and significant down-regulated activity of PPARγ, SREBP2, and SCD gene expression when compared to the HFDC group. In addition, the mice treated with 20mg/kg and 30 mg/kg of ABZ methanolic extract showed a significant reduction of TNFα and IL-6 and significant increase of adiponectin and leptin compared to the HFDC. In conclusion, the antioxidant properties of 20mg/kg to 30 mg/kg methanolic extract of ABZ bark is able to avert obesity by regulation of the genes and adipocytokines involved in lipid metabolism.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

AKTIVITI ANTI-OBESITI DAN ANTIOKSIDAN EKSTRAK METANOL KULIT Albizia myriophylla (L. BENTH) PADA TIKUS OBES

Oleh

AZMAH SA’AT

Julai 2017

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Di peringkat global dan di Malaysia, obesiti merupakan antara penyebab morbiditi dan kematian di peringkat awal berserta kos rawatan yang mahal. Aktiviti antioksidan dalam tumbuhan herba telah dicadangkan sebagai salah satu daripada mekanisma untuk mengatasi masalah obesiti. Oleh itu, kajian ini mengkaji aktiviti antioksidan Albizia myriophylla (ABZ) yang lebih dikenali sebagai tebu gajah di Malaysia. Kesannya kepada anti-kegemukan melalui ujian in vivo dalam obesiti yang dirangsang melalui diet lemak tinggi di kalangan tikus yang terdiri dari kumpulan kawalan normal (NC), kumpulan diet lemak tinggi (HFDC), kumpulan diet lemak tinggi yang di rawat dengan ekstraksi metanol kulit ABZ pada aras 10 mg/kg, 20 mg/kg dan 30 mg/kg turut dikaji selama 5 minggu. Aktiviti antioksidan ekstraksi metanol dan pecahan ekstrak yang diperoleh daripadanya seperti hexana, klorofom, etil asetat, butanal dan sisa pecahan akues daripada kulit ABZ telah dilakukan dengan menggunakan analisis kimia secara in vitro. Dalam ujian memerangkap radikal DPPH (1,1-diphenyl-2-picrylhidrazyl), ujian memerangkap radikal ABTS (2-2’-azinobis 3-ethyl-benzothiazoline-6-sulfonic acid) dan ujian pengurangan aktiviti terhadap ion ferus (FRAP), jumlah keupayaan antioxidan didapati berbeza-beza dalam fraksi yang berlainan. Nilai IC₅₀ daripada tiga kajian ini menunjukkan bahawa ekstraksi metanolik kulit ABZ menunjukkan nilai IC₅₀ yang terendah untuk ketiga-tiga ujian, dibandingkan dengan ekstraksi yang lain, serta menunjukkan aktiviti antioksidan yang tertinggi. Dapat-dapat di atas menunjukkan bahawa ekstrak metanolik kulit ABZ adalah sumber antioxidan semulajadi yang berpotensi dan telah dipilih untuk digunakan dalam kajian anti-obesiti. Penilaian berat badan tikus, kajian sellulariti adipos, dan kadar ketinggian kolestrol, trigliserida, lipoprotin berketumpatan rendah (LDL), aras malondialdehyde dalam otot tikus gemuk, serum aspartate transaminase (AST) dan serum alanine transaminase (ALT) turut dikaji. Hasilnya telah menunjukkan kehilangan berat yang signifikan berserta penurunan ketara dalam bilangan dan saiz sel-sel adipos pada tikus-tikus yang telah diberikan 20 mg/kg dan 30 mg/kg ekstraksi metanolik kulit ABZ. Tambahan pula, berikut rawatan 20 mg/kg dan 30 mg/kg ekstrak metanol dahai ABZ, terdapat pengurangan kolesterol, TG dan LDL yang
ketara dilihat dalam kumpulan-kumpulan ini. Di smping itu, pengurangan AST, ALT
dan malondialdehyde yang ketara dalam kumpulan 20 mg/kg dan 30 mg/kg
dibandingkan dengan kumpulan HFDC, menggambarkan ekstraksi metanolik kulit
ABZ adalah selamat digunakan dalam model tikus. Kesan pengawalaturan ekstrak
metanol ABZ pada gen yang melibatkan lipase hormon sensitif (HSL), peroksosim
resorpti proliferator-diaiktikan α dan γ (α PPAR dan PPAR γ), sterol peraturan unsur
mengikat protein-2 (SREBP2) dan stearoyl-Koenzim A-desaturase (SCD) gendan
adipositokin termasuk tumor nekrosis faktor-α (TNFα), interleukin-6 (IL-6),
adiponectin dan leptin yang terlibat dalam metabolisma lemak juga telah dikaji. Tikus
gemuk yang dirawat dengan 30 mg/kg ekstrak metanol ABZ memenjukkan aktiviti
yang ketara kepada kenaikan kadar aktiviti PPARα dan penurunan kadar aktiviti
PPARγ, SREBP2, dan SCD ekspresi gen apabila dibandingkan dengan kumpulan
HFDC. Di samping itu, kumpulan yang dirawat dengan 20 mg/kg dan 30 mg/kg
menunjukkan pengurangan ketara TNFα dan IL-6 serta menyaksikan peningkatan
ketara adiponektin dan leptin berbanding dengan kumpulan HFDC. Kesimpulannya,
sifat-sifat antioksidan 20 mg/kg kepada 30 mg/kg ekstrak metanol kulit ABZ mampu
mengelakkan kegemukan dengan pengawalaturan gen dan adipositokin yang terlibat
dalam metabolisma lemak.
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I certify that a Thesis Examination Committee has met on 20 July 2017 to conduct the final examination of Azmah binti Sa'at on her thesis entitled "Anti-Obesity and Anti-Oxidative Effects of Methanolic *Albizia myriophylla* (L.Benth) Bark Extract in Obese Mice" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ABTS</td>
<td>2-2′-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)</td>
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<tr>
<td>ABZ</td>
<td><em>Albizia myriophylla</em></td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate kinase</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate kinase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate transaminase</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle Threshold</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1 picrylhydrazyl</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric reducing antioxidant power</td>
</tr>
<tr>
<td>GP</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HDL</td>
<td>High-Density Lipoprotein</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>HFDABZ1</td>
<td>High-fat diet mice treated with ABZ 10 mg/kg</td>
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<tr>
<td>HFDABZ2</td>
<td>High-fat diet mice treated with ABZ 20 mg/kg</td>
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<tr>
<td>HFDABZ3</td>
<td>High-fat diet mice treated with ABZ 30 mg/kg</td>
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<tr>
<td>HFDC</td>
<td>High-fat diet control</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>HKG</td>
<td>House keeping gene</td>
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<tr>
<td>HMG-CoA-R</td>
<td>Hydroxy-methylglutaryl-Coenzyme A reductase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase enzyme</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone-sensitive lipase</td>
</tr>
<tr>
<td>IC</td>
<td>Inhibition concentration</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
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<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>mTOR</td>
<td>Mechanism target of Rapamycin</td>
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<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
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<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
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<tr>
<td>NC</td>
<td>Normal control</td>
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<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>pAMPK</td>
<td>Phosphorylation of Adenosine monophosphate-activated protein kinase</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor alpha</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative reverse transcription PCR</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>SCD</td>
<td>Stearoyl-Coenzyme A-Desaturase</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
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<tr>
<td>SOD</td>
<td>Sodium dismutase</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>SREBP</td>
<td>Sterol Regulatory Element-Binding Protein</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
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<tr>
<td>TG</td>
<td>Triglyceride</td>
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<tr>
<td>TMB</td>
<td>3,3,5,5′-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TSFA</td>
<td>Total saturated fatty acid</td>
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<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
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<td>WAT</td>
<td>White adipose tissue</td>
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CHAPTER 1
INTRODUCTION

Obesity is fast becoming a major health concern throughout the world (Hurt et al., 2010). The predisposing factors of obesity have multi-factorial facets involving diet, sedentary lifestyle, dysregulation of the genes and adipocytokines involved in the lipid metabolism (Valavanis et al., 2010). The health burden from obesity is largely driven by an increased risk of type 2 diabetes, cardiovascular diseases, and several forms of cancer such as colon and breast cancer (Renehan et al., 2008). The medical costs of obesity represent the monetary value of health-care resources devoted to managing obesity-related disorders, including the costs incurred by excess use of ambulatory care, hospitalisation, drugs, radiological or laboratory tests, and long term care (Guh et al., 2009). Therefore, obesity proves to be a challenge in the prevention and treatment of the foreseeable conditions stated above.

Obesity is closely related to increasing inflammatory environment and enhanced oxidative stress conditions in the cells (Savini et al., 2013). Obesity consisting of adipose cells accumulation is generally known as a crucial energy supplier and storage and was recently acknowledged as an important endocrine tissue which is actively involved in lipid metabolism (Wang et al., 2014). Undeniably, the endocrine function of the adipose tissue plays a major role in the energy balance with the involvement of the adipocyte derivatives involving the pro-inflammatory and anti-inflammatory adipocytokines (Wang and Huang, 2015). When the generation and release of pro-inflammatory adipocytokines such as TNFα and Il-6 exceed the production of anti-inflammatory adipocytokines such as adiponectin and leptin, systemic inflammation and obesity associated metabolic disorders occurs (Johnson et al., 2012). Hence, the development of obesity is related to the chronic low-grade inflammatory condition (Wang et al., 2014).

Dietary consumption of nutrients rich with anti-inflammatory bioactive components from plants which are high with antioxidant activities such as omega-3 fatty acids and polyphenols, has demonstrated anti-inflammatory activity with reduced inflammation in breast cancer tissue and obesity (Kalupahana et al. 2011; Siriwardhana et al., 2013; Savini et al., 2013; Khan and Mukhtar, 2013; Jeong et al., 2016). A review article by Esfahani et al. (2011), stated that consumption of mixed fruits and herbal supplements, considerably reduces the oxidative stress activity and elevates the antioxidant levels in the systemic circulation. This evidence supports the dietary guidelines of Malaysians which emphasize the importance of a diet rich in high fiber diet consisting of fruits and vegetables, to prevent the development of obesity (Tee, 2011).

Several researches stated that polyphenols consumption prevents development of obesity via numerous probable mechanism which includes reducing adipogenesis via suppressing the growth and differentiation of adipocytes by inhibiting the PPARγ and SREBP2 gene expression (Mayoral et al., 2015; Moseti et al., 2016), reducing the
inflammatory activity and attenuating the oxidative stress condition via stimulation of the PPARα gene expression which reduces the anti-inflammatory precursors namely the TNFα and IL-6, and increases the anti-inflammatory adipocytokines which are the adiponectin and leptin (Jung and Choi, 2014; Jia et al., 2016) and also by reduction of the cholesterol synthesis via the suppression of SREBP2 gene (Taylor et al., 2011). In addition, the down regulation of SCD gene will help in stimulating the insulin sensitivity action, thus producing effective energy utilization (Karahashi et al., 2013).

Among the most common pharmacological drugs concurrently used as an anti-obesity agent in the clinical settings are orlistat, which inhibits the gastric and pancreatic lipases thus, reducing the lipid absorption from the gut and sibutramine, which acts as an oral anorexiant (Kang and Park, 2012). The high rate of side effects such as myocardial ischemia specifically associated with sibutramine, has been the reason for the drug to be withdrawn from the market in several countries (Kang and Park, 2012). This side effect seen in the current synthetic anti-obesity drug stresses the necessity of finding a suitable replacement of alternative with a natural or herbal product.

1.1 Significance of Study

Natural or herbal products have shown great promise as novel drug leads (Cragg and Newman, 2013). Numerous herbal products have been used as an anti-obesity agent. For example, astaxanthin, a dietary carotenoid reduces lipid accumulation in mice through the inhibition of PPARγ and activation of PPARα (Jia et al., 2016). Consequently, for a more efficient treatment and control of obesity or weight gain and to satisfy unmet medical needs, there is a necessity to develop new anti-obesity agents that specifically up-regulate the PPARα expression and down-regulate the PPARγ and SREBP2 expression. To explore this possibility, in this study, the researcher has used the ABZ bark and its various fractions extracted through maceration process, and tested its antioxidant activities and gene transcription studies.

In Peninsular Malaysia, ABZ or tebu gajah aqueous bark extracts was used as a tonic drink which was mixed with other herbal supplementation. It was taken as an alternative medication for the treatment of diabetes. Following this, a study by Saat et al. (2012) has proven that the ABZ aqueous bark extract had significant hypoglycemic activity in streptozotocin nicotinamide induced diabetic rat. As diabetes is closely associated with obesity, with similar pathogenesis, ABZ bark extract may have a potential anti-obesity effect, as well, via indirectly from the antioxidant or free radical scavenging activity. A patent in the United States had used ABZ bark to prevent obesity but not as an anti-obesity agent, as the bitter after taste of saponin thwarts over eating (Patent Application 20040146468).

In the present study, the effect of high fat diet on the liver of the obese mice was also evaluated. The levels of liver enzymes which are AST and ALT, along with the hematoxylin and eosin staining of the hepatocytes of the mice were done. The safety of consumption of the selected doses of ABZ bark in mice was investigated through
evaluation of the obese mice liver. The histology of the kidney was not done due to financial constraint.

Thus, the significance of this study is in the hope that the anti-obesity action of Albizia myriophylla bark extract will provide an alternative and play a significant role in body weight management.

1.2 Hypothesis

The antioxidant activity of A. myriophylla will regulate the adipogenesis pathway by controlling the adipokines and genes involved in the lipid metabolism, thus leading to a reduction in the number and size of adipocyte cells, eventually controlling obesity and is safe in mice model.

1.3 General Objective

The main objective of this study was to investigate the potential anti-obesity activity of A. myriophylla bark extract in lipid metabolism and gene regulation of obesity-induced mice.

1.4 Specific Aims

1. To determine the polyphenol composition and content in ABZ bark extract and its antioxidant activity.
2. To analyse the body composition, adipocyte cellularity and blood serum parameters in obesity-induced mice given ABZ bark extract.
3. To evaluate the mRNA expression patterns of different genes in the liver.
4. To evaluate the effect of ABZ bark extract in obesity-induced mice liver.
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