The role of beluntas (pluchea indica less.) leaf extract in preventing the occurrence of fibroblasts hyperproliferation: An *in vitro* preliminary study

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Abstract

Beluntas (Pluchea indica Less.) is a herbal plant which contains variety of benefits. Quercetin, one of flavonoid, is the most bioactive agent in beluntas leaf. Collagen inhibition by quercetin may modulate extracellular matrix deposition and inhibit the formation of hypertrophic scar. This was an in vitro study with senescent fibroblasts to determine the role of beluntas leaf extract in preventing the occurence of fibroblasts hyperproliferations. There were 4 groups were stained by anti-collagen I antibodies and secondary antibody. Flowcytometry analysis was done to measure the fibroblasts density. Anova test was performed with a value of p=0.000 (p<0.05). A post hoc analysis showed significant differences in the average decrease of fibroblasts that absorbs staining anti-collagen I antibody treatment group compared with the control group. There were significant effects of beluntas leaf extract in preventing the occurrence of fibroblasts hyperproliferations. Beluntas leaf extract with a concentration of 80 mol/L had the most significant effect on the fibroblasts density. Thus beluntas leaf extract has the ability in preventing the occurrence of fibroblasts hyperproliferation.

Introduction

In the last few decades, various scientific studies involving herbal medicinal plants has evolved. Plants remain as important source of new drugs. Plants have been utilized as medicine for thousands of years. In search for better treatment option, many patients have turn to alternative medicine in the hopes of identifying more natural substances with less toxicity but equal efficacy. The World Health Organization (WHO) study reported that the use of herbal medicinal plants has low side effects, cheaper, and easily obtainable.[1,2] Beluntas (Pluchea indica Less.) has the bioactive content as phytochemicals mainly flavonoids, triterpenoids, phenols, sterols, glycosides and essential oils.[3] Flavonoids are divided into several classes one of which is flavonols containing quercetin, the most compound in beluntas.[4] Quercetin was previously reported has antioxidant and antifibrotic activity.[2]

Normal fibroblasts work to regulate synthesis collagen in the dermis.[5] Dermis composed connective tissue contains fibroblasts and collagen. The role of fibroblasts in producing extracellular matrix (ECM)-forming agents is crucial not only as the development and maintenance of normal tissue structures, but also improvements and reshuffle in the wound healing process.[6] Fibroblast fibers are used in vitro studies as a model because fibroblasts are the primary producer of collagen in the dermis layer, especially type I collagen, to study wound healing and scar. Collagen inhibition by quercetin may modulate extracellular matrix deposition and inhibit the formation of hypertrophic scar.[7,8] Since there has never been reported before, the authors hypotezised that there are role in beluntas leaf extract for preventing the occurence of fibroblasts hyperproliferation.

Materials and Methods

Invitro study was conducted from September 2016 to February 2017 in Dr. Moewardi General Hospital, Dermama Biotechnology Laboratory Surakarta and the Agricultural and Development Research Institute of Spices and Medicines Ministry of Agriculture (BALLITRO) Bogor. Beluntas leaf extract was taken from Pawana kepurun Indonesia company in Yogyakarta. The antibodies anti-collagen I was purchased from cambridge, United Kingdom. The sample study used fibroblast cultures obtained from human placenta. The inclusion criteria was placenta obtained from woman who gave birth to a healthy baby, HBsAg negative examination, non-reactive anti HIV test, and was willing to sign informed consent while the exclusion criteria was a woman with a genetic disease associated with collagen abnormalities and unwilling to participate in the study. Beluntas leaf extract is an extract obtained from beluntas plant which is divided into 3 concentrations, 20 µmol / L, 40 µmol / L, and 80 µmol / L. Type I collagen deposition was calculated using flowcytometer over cultured fibroblasts with starvation of 1% fetal bovine serum (FBS) and treated with 3 concentration of beluntas leaf extract. The measurement result was the percentage of antibodies anticolagen I absorbed by senescence fibroblasts. This study was approved by Dr.Moewardi General Hospital ethical committee.

The difference of type I collagen deposition from the control group and the treatment groups was analyzed by one-way ANOVA and post hoc test. Primary antibodies are used to bind to collagen type I specific collagen I protein antigen. Secondary antibody is intended for immunolabeling. This study used primary antibodies Anti-Collagen I antibody [5D8-G9] ab23446 (Abcam®) and secondary antibody Goat Anti-Mouse IgG H & L (Alexa Fluor® 488) (Abcam®). Primary Antibodies Anti-Collagen I antibody [5D8-G9] ab23446 (Abcam®) has been used in studies of human type IV collagen, whereas the use of Goat Anti-Mouse IgG H & L (Alexa Fluor® 488) (Abcam®) has also been used as secondary antibodies in the study of tight junctions (TJs) and adherent junctions (AJs) of adenocarcinoma epithelial cells in humans. Although derived from mice, these antibodies have cross-adsorption with IgG and human serum.

Results

The culture of senescence fibroblasts showed that giving beluntas leaf extract had an effect on decreasing the amount of

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fibroblasts which absorb type I collagen antibody. The higher concentration of beluntas leaf extract given, the less the number of cells that absorbed type I collagen antibody shown by the decreased of fibroblasts density (Figure 1). Flowcytometry analysis showed that the number of detected cells absorbed decreased along with the large amount of beluntas leaf extract given (Figure 2).

The normality test between groups after flowcytometry analysis with Shapiro-Wilk showed p value > 0.05 in all samples, therefore that the sample in this study is homogeneous. Levene test was done to analyze the variance between groups, and the result was p>0.05 meaning this study had the same variance so that it met the requirement of ANOVA test. The result of ANOVA test on four different treatments showed the value of p<0.05. It could be assumed that there was a significant effect on the different concentrations of beluntas extracts to the deposition of senescence fibroblasts (Table 1).

Discussion

Based on the results of phytochemical tests of the Agricultural Research and Development of Indonesian Spice and Medicinal Plant Research Institute Bogor, leaf extract qualitatively contains flavonoids. In the ANOVA extract test of leaf beluntas with concentration of 20 µmol / L, 40 µmol / L, and 80 µmol / L was shown to have an effect on inhibiting the fibroblasts density on senescence fibroblast cultures and was statistically significant with p=0.000 (p<0.05) so that the research hypothesis is accepted. Among the treatment groups, the concentration of beluntas leaf extract of 80 µmol / L had the greatest inhibitory effects on fibroblasts density. It was concluded that the concentration of beluntas leafs 80 µmol / L was the highest concentration to preventing the fibroblasts hyperproliferations and was statistically significant p=0.000 (p<0.05). This is in accordance with previous research which stated that this inhibitory effect has the maximum effect at 80 µmol / L concentration with lethal dose was achieved at an extract concentration of 100 µmol / L. The decrease in fibroblasts hyperproliferations in the administration of beluntas leaf extract is made possible by the content of quercetin as flavonoids in beluntas which gives effect on inhibition of collagen synthesis, by interfering the biosynthesis of collagen precursor molecules known as procollagen.[9] It has always been very easy to get fibroblasts to multiply in vitro, they are commonly accused of swamping the growth of other kinds of cells in a culture.[10] Normal fibroblasts work to regulate synthesis and degrade collagen in the dermis. The senescence fibroblasts are fibroblasts that lose their ability to produce collagen, procollagen and Tissue Inhibitors of Matrix Metalloproteinases (TIMP). Hence senescence fibroblasts mimick the patological process of chronic inflammatory response.[11] The increased presence of type III collagen at the beginning of the tissue repair phase, is physiologically perceived as the onset of miofibroblasts early in the tissue repair phase. However, in the pathological process, there is a significant increase in the number of type III collagen in the whole process, which can result in the formation of scar in the normal dermis.[12,13] Increased collagen synthesis is a major problem especially in the fibrotic tissue repair response due to cellular damage and / or chronic inflammatory response. In inflammatory lesions, fibrin may serve to induce local

Table 1. The results of ANOVA test measurements of the independent and dependent variables.

Groups	Fibroblasts density	p
Control	1.5%	0.000
Beluntas 20 µmol/L	1.3%	0.000
Beluntas 40 µmol/ L	1.0%	0.000
Beluntas 80 µmol/ L	0.7%	0.000

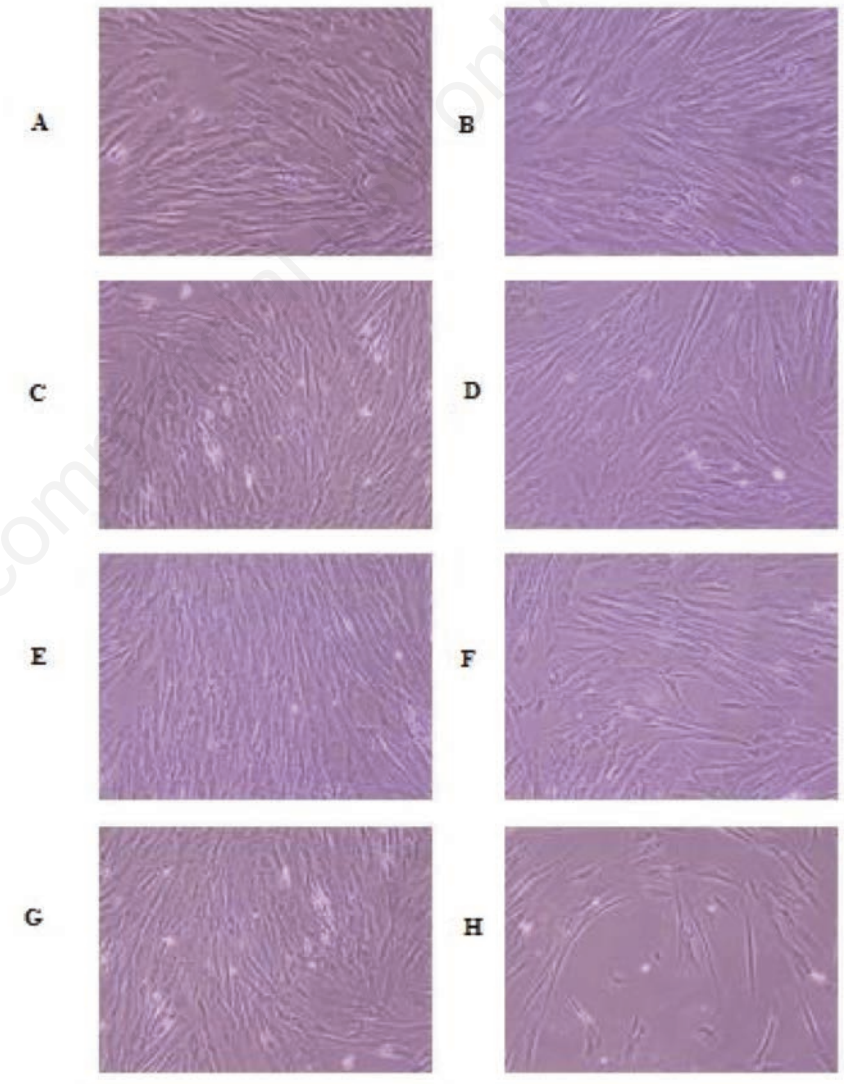

Figure 1. The distribution of fibroblasts in starvation cultures. A-B. Control groups. C. Group 2 before treatment. D. Group 2 after giving beluntas leaf extract at concentration of 20 mol / L. E. Group 3 before treatment. F. Group 3 after extract of beluntas leaves with concentration of 40 mol / L. G. Group 4 before treatment. H. Group 4 after giving beluntas leaf extract with concentration of 80 mol / L. The density of the cell decreases as the concentration of beluntas leaf extract increases

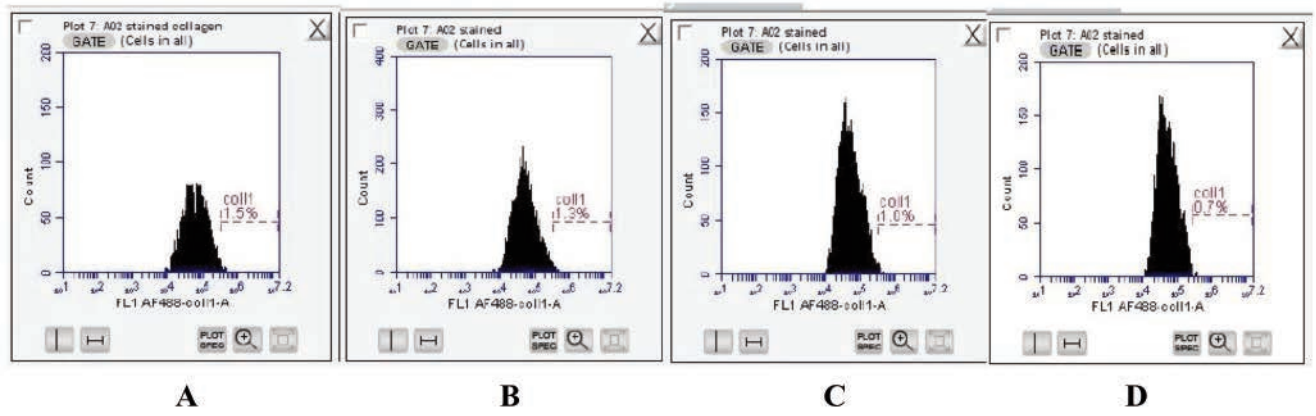

Figure 2. A. The results of flowcytometric analysis of group 1 (control), B. group 2 (treatment with beluntas extract with 20 µmol / L concentration), C. group 3 (treatment with beluntas extract with concentration of 40 µmol / L), D. group 2 (treatment with extract beluntas with a concentration of 80 µmol / L).

fibroblast response. This becomes important in the early stages of increased collagen synthesis after tissue inflammation. The amount of fibrin will be determined by the location and nature of the damaged cell, and the fibrin will again stimulate the fibrotic response. Fibroplasia will be reduced by the decrease in fibrin deposition, by increased fibrinolysis, resulting from decreased and stabilized collagen synthesis, or due to increased collagen degradation.[12,14,15] The limitation of this study is the calculation of beluntas leaf extract concentration was based on relative molecular mass and quercetin content in 100 grams of dried extract of beluntas leaf and not on pure quercetin extract.

Conclusions

There was significant effect of beluntas leaf extract in preventing the occurrence of fibroblasts hyperproliferations. Beluntas leaf extract with a concentration of 80 mol / L had the most significant effect on the fibroblasts density. Our study revealed that beluntas leaf extract has the ability in preventing the occurrence of fibroblasts hyperproliferation.This is an invitro preliminary study, thus further study needs to be performed invivo.

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