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GC-MS Analysis and In vivo Anti-Ageing Activity of Indonesian *Ficus carica* Ethanolic Extract

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ABSTRACT

Many researchers have conducted analysis of GC-MS and the biological activity of *Ficus carica*, however, there has not been research on the phytochemical components and anti-ageing activity of ethanol extracts originating from Indonesia. The objective of this study is to investigate GC-MS analysis and in vivo anti-ageing activities of these particular ethanolic extracts. Materials and Methods: The ethanolic extract was analyzed using GC-MS and tested for the anti-ageing effect of *F.carica* leaves on mice treated with D-galactose. D-galactose (100 mg/kgBW/day) was subcutaneously injected daily, for 42 days, to build an ageing model. The extract of *F.carica* leaves (50, 150, and 450 mg/kgBW/day) was administered daily for 42 days, beginning on the second day. Spectrophotometry was used to measure the malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, and glutathione peroxidase (GSH-Px) activity. Results: The results showed that both moderate-dose and high-dose extracts of *F.carica* leaves enhance SOD, GSH-Px, and Catalase, and reduce MDA content significantly ($P < 0.05$). The results obtained revealed the presence of about 13 phyto-compounds, along with various potential bioactive compounds, which could be useful for both industrial and pharmaceutical purposes. Conclusion: This study confirms that the ethanolic extract of *F.carica* leaves and its mechanisms can be associated with increased antioxidant enzymes, such as SOD and GSH-Px, as well as decreased production of MDA, and enhanced scavenging of free radicals.

Keywords: Medicinal plants; *Ficus carica*; Moraceae, ageing

INTRODUCTION

Ultraviolet (UV) radiation is one of the dominant causes of aging. UV rays stimulate reactive oxygen species ROS), resulting in the accumulation of oxidative products, leading to oxidative stress. The damage caused by ROS causes aging (Yin and Chen, 2005). Oxidants attack the unsaturated fatty acids in the body, causing a chain reaction known as lipid peroxide. The process results in the breakdown of fatty acids into various toxic compounds like malondialdehyde (MDA). Antioxidants can lower the concentration of MDA in the cell membranes (Halliwel and Gutterdge, 1998). These are like the body's defense mechanism. Antioxidants and other enzymes reduce ROS damage by neutralizing and resisting toxic substances or free radicals, inhibiting oxidant creation (Jin and Yin, 2012). Antioxidants in the cells are divided into two groups:

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enzymatic and non-enzymatic. Enzymatic antioxidants, also called preventive antioxidants, including superoxide dismutase, catalase, and glutathione peroxide. Non-enzymatic antioxidants, chain-breaking antioxidants, include vitamin C, vitamin E and beta-carotene (Halliwel and Gutterdge, 1998). The interaction between free radicals, antioxidants, and cofactors is important in understanding health, the aging process, and aging-associated diseases. It's important that the body's endogenous antioxidant system matches the amount of oxidative stress induced by free radicals. If the amount of free radicals is higher than the protective antioxidants and cofactors, the oxidative damage will accumulate, affecting the aging process and aging-associated maladies like cardiovascular diseases, cancers, neurodegenerative disorders, and other chronic conditions (Ames et al., 1993). the *D-galactose*-induced mouse aging model is recognized by domestic and international researchers and widely used in the field of anti-aging medical research (Song et al., 1999). The degree of aging in the model was close to 16–24 , and all changes were consistent with natural aging (Ho et al., 2003). A study conducted by Deng *et al*. (2003), demonstrated that long-term D-galactose-induced mice had increased production of free radicals, characterized by higher MDA levels and lower levels of SOD activity. The administration of D-galactose may also result in the decreased function of the vital organs, such as the liver, heart, kidneys, and thymus. A similar study conducted by Oroojan *et al*. (2016), showed that the long-term induction of D-galactose can result in a decreased weight of the organs as aging occurs. *Ficus carica* Linn, (Moraceae) is commonly known as an edible fig or tin, its local name. The leaves, roots, fruits, and latex of the plant are medicinally used in different diseases.

Fig 1. *Ficus carica* Linn (Moraceae)

Several studies have demonstrated the fatty acid content of *F.carica*, dentified as myristic, pentadecylic, palmitic, margaric, cis-10-heptadecenoic, stearic, oleic, elaidic, linoleic, arachidonic, heneicosanoic, behenic, tricosylic and lignoceric (Jeong and Lachance, 2001; Joseph and Raj, 2011). The research conducted by Marreli *et al*. (2014) found that the leaves of *F.carica* have higher

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antioxidant activity than the other parts of the plant and that they contain unsaturated fatty acids (Marreli et al., 2014). The susceptibility of fatty acids to oxidation is thought to be directly dependent on their degree of saturation (Richard et al., 2008).In this study, mice were injected with D-galactose continuously during the predetermined period. In the catalysis process of aldose reductase, the galactose is reduced to galactitol. Galactitol can no longer be metabolized and accumulates in the cells, causing changes in their osmotic pressure. The free radicals obtained from *D-galactose* being metabolized can cause aging by increasing ROS and decreasing antioxidant enzyme activity (Song et al., 1999). In addition, D-galactose accumulation in animal tissues can react with amino acids and peptides, forming the *Advanced Glycation End* (AGE) product. This plays a role in the aging process, and the pathogenesis of aging-associated diseases, such as diabetes, arteriosclerosis, nephropathy, and Alzheimer's (Ho et al., 2003). *Ficus carica* L. is a widely known and commonly consumed Mediterranean plant. Its fruit does not contain fat or cholesterol; it is full of vitamins and is a good source of dietary fiber and mineral elements. The objective of this research is to explore the long-term effects of Indonesian *F.carica* leaf extracts on *D-galactose*-induced mice.

MATERIAL AND METHODS

Plant materials:F.carica was collected in July 2016 from Bogor, West Java, Indonesia. The plant was authenticated by Dr. Joen Setijo Rahajoe from Biological Research Center LIPI. The specimen vouchers were deposited to the herbarium at Department of Pharmacognosy, Faculty of Pharmacy, Pancasila University.

*Preparation of extracts:*502.5g of powdered simplicia fig leaves was put into two vessels, and then extracted via kinetic maceration at room temperature using 70% ethanol solvent. The re-maceration was performed seven times, with a total solvent of 16 L. The obtained maceration was collected and concentrated using a rotary evaporator at 40°C. The condensed extract obtained totaled 130.1g, resulting in a DER-native of 3.8624 with 25.89% yield.

The in vivo anti-aging test: Adult Swiss mice (*Mus musculus* Linn) were obtained from the animal facilities of Pancasila University. They were kept in cages under standard conditions of light (12 h with alternative day and night cycles) and temperature (25± 1°C). The mice were housed with access to commercial rodent feed and water *ad libitum*. The investigational protocol was approved by the local Ethical Committee in Animal Research.

Thirty healthy mice were divided into 6 groups, each consisting of 5 members. All were dissected on the 43rd day, as follows: 1) the normal group, induced by aquadest for 42 days; 2) the negative control group, induced by D-galactose with a dosage of 100 mg/kg subcutaneously for 42 days; 3) positive control, group induced by D-galactose with a dosage of 100 mg/kg subcutaneously and by vitamin C with a dosage of 100 mg/kg by BW orally for 42 days; 4) a group induced by D-galactose with a dosage of 100 mg/kg subcutaneously and by a preparation of fig leaf ethanol extract with a dosage of 50 mg/kg by BW orally for 42 days; 5) a group induced by D-galactose with a dosage of 100 mg/kg subcutaneously and by a preparation of fig leaf ethanol extract with a dosage of 150 mg/kg by BW orally for 42 days; and 6) a group induced by D-galactose with a dosage of 100 mg/kg subcutaneously and by a preparation of fig leaf ethanol extract with a dosage of 450 mg/kg of BW orally for 42 days (Cui et al., 2006). On the 43rd day, the mice were dislocated, dissected and then taken for their vital organs (liver, heart, and kidneys). The vital organs were then put into a glass beaker, which was previously filled with 0.9% NaCl. The glass beaker was then weighed. The weight was obtained by calculating the difference between the weight of the glass beaker before and after the addition of vital organs. The organ index ratio was calculated by:

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Ratio (mg/g) = vital organ weight(mg)/body weight of mice(g)

Biochemical analysis: SOD activity was measured using the xanthine oxidase method. The activity of catalase was analyzed by measuring the absorbance of H_2O_2 at 240 nm. GSH-Px activity was determined by quantifying the catalyzed reaction rate of H_2O_2 and GSH at 420 nm, spectrophotometrically. Lipid peroxide was tested by measuring the MDA content at 532 nm (Cui et al., 2006; Weyder and Culler, 2010).

GC-MS analysis The GC-MS analysis was carried out by an Agilent system equipped with a mass spectrometer and split/splitless injection system. The GC was fitted with an HP-5MS capillary column (30 m X 250m; film thickness: 0.25m). The temperature program was as follows: injector at 280°C, an initial oven temperature of 50°C, then increased to 25°C/min to 300°C and held for 10 min. Helium was used as the carrier gas at a pressure of 17.69 psi, with a flow of 2.1 ml/min. Samples were dissolved in methanol, and 1 µl aliquot was injected automatically. MS spectra of separated components were identified based on WILEY and NIST Libraries for botanical compounds.

*Statistical analysis:*The data were expressed as mean ± SD. Dunnett's t-test was used for comparing differences between the treated and aged groups, and the differences were considered significant at $p<0.05$.

RESULTS

The identification of ethanol extract component of *F.carica* leaves was made by comparing the fragmentation pattern of the mass spectrum with the fragmentation pattern of the reference compound. The result data of the analysis of compound components contained in ethanol extract of *F.carica* leaves using the databank WILLEY9THN 08.L is shown in Table 1. The major constituents were ethyl-hexadecanoate (RT 19.386), n-hexadecanoic acid (RT 19.252), 9-octadecenoic acid (RT 20.914), and 9,12,15-octadecatrienoic acid (RT 21.164). Before experimentation, all mice that would be used for the experiment were adapted in each group for 7 days with the same treatment of foods, drinks, and cages. After 7 days of adaptation, the calculation of the weight of each mouse was conducted, and all mice were then given for treatment based on the group that had been predetermined for 42 days. The calculation of body weight during treatment was conducted on the 12th, 24th, 36th and 42nd day of each time before the induction of D-galactose and test preparation (Table 2). Table 3 shows that the average MDA level of the aging group (4.69±2.45) increased compared with the normal group (1.61±1.52) and the treatment with the dosages of 50, 150 and 450 mg/kg of BW (1.98±1.05, 1.78±1.45 and 1.94±1.85). This study is in line with the research conducted by Chen et al. (2016), which showed that the induction of D-galactose with a dosage of 500 mg/kg daily for 8 weeks canincrease the MDA level. This is due to the continuous induction of increased D-galactose in the body. D-galactose can be catalyzed through aldose reductase into galactitol, which cannot be further metabolized and accumulates in cells. Table 4. shows the SOD activity in serum of mice. At a dosage of 150 mg/kg per BW, the ethanol extract of fig leaves could increase the activity of SOD enzymes proportionally to the ascorbic acid treatment group. This result shows that the higher the dosage of ethanol extract, the greater the increase in SOD activity. However, at a higher dosage of 450 mg/kg per BW, the SOD enzyme activity decreased. After 42 days of treatment, the mice were dislocated and dissected to weigh their livers, kidneys, and hearts on the 43rd day. The data on organ weight is shown in Table 4.

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No	Retention Time	Name of Compound	Molecular Formula	Molecular Weight	Qual
1	3,084	Propanoic acid	CH_3CH_2CO OH	74.0785	59
2	3,293	Methoxyformamide Glycin	$C_2H_5NO_2$	75,066	45
3	13,558	1,5-Naphthalenediol	$C_{10}H_8O_2$	160,1693	90
4	17.999	2H-Furo[2,3-H]-1-benzopyran-2one (Angelicin)	$C_{11}H_6O_3$	186,1635	94
5	20.395	5-methoxy-6,7-furanocumarin (bergapten)	$C_{13}H_8O_4$	216,192	97
6	19.386	N-hexadecanoic Acid	$C_{16}H_{32}O_2$	284	99
7	19.252	Xanthotoxin metoxalen	$C_{12}H_8O_4$	216,1895	94
8	20.511	Amyrolin (Seselin)	$C_{14}H_{12}O_3$	228,247	91
9	20.616	Phytol	$C_{20}H_{40}O$	296	98
10	20.914	9-Octadecenoic acid Oleic acid	$C_{18}H_{36}O_2$	284,4772	99
11	21.088	Linoleic acid	$C_{18}H_{32}O_2$	280,432	97
12	21.164	9,12,15-octadecatrienoic acid	$C_{10}H_{32}O_2$	292	99
13	24.084	2-Dodecylcyclohexanone	$C_{28}H_{34}O$	266,469	41
14	26.614	bergaptol	$C_{11}H_6O_4$	202,165	64
15	27.535	Squalene; spinacen	$C_{30}H_{50}$	410,718	99
16	28,380	glycerol	$C_3H_8O_3$	92,094	60
17	33.152	Dl-alpha-tocopherol	$C_{29}H_{50}O_2$	430,717	97
18	38.562	Beta-sitosterol	$C_{29}H_{50}O$	414,718	95
19	39.757	Beta-amyrin	$C_{30}H5_0O$	426,729	98
20	41,384	Alpha-amyrin	$C_{30}H_{50}O$	426,729	42

Table 1: GC-MS analysis revelaled the presence of phyrochemical components in ethanol leaves extract of *Ficus carica*

Group	Dosage (mg/kg/day	1st day Wt (g)	7th day Wt (g)	% Δ^7	36th day Wt (g)	% Δ^{36}	42rd day Wt (g)	% Δ^{42}
Norma 1	-	20.5±1.35	22.5±1.25	9.75	26.5±1.35	11.50	27.5±1.35	13.24
Aged	100	22.2±2.12	28.2±1.12	27.02	29.8±2.12	28.45	28.0±2.12	30.20
Extract Ficus	50	20.9±1.45	21.9±2.45	4.78	23.9±1.35	8.50	25.9±1.25	10.20
	150	21.5±1.56	22.5±1.66	4.66	26.5±1.16	9.70	28.5±1.51	12.30
	450	20.6±1.5	23.6±2.51	14.56	25.6±1.51	15.50	32.6±1.23	17.30
Ascorbic acid	100	22.5±1.75	25.5±1.85	13.33	27.2.5±1.35	14.40	32.5±1.15	19.90

Table 2: Body weights

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Group	Dosage (mg/kgBW)	CAT activity Ug/protein	GSH-Px activity (Unit/protein)	MDA content (nmol/protein)	SOD activity (Unit/mL)
Normal	-	265±9.5	925.4±10.5	1.61±1.52	123.4±1.5
Aged	100	225.5±15.4	620.6±15.4	4.69±2.45*	105.5±2.6
Extract Ficus	50	235.4±20.5	695.4±16.5	1.98±1.05	105.4±2.4
	150	273.25±17.5*	895.5±20.4*	1.78±1.45	120.8±2.5*
	450	238.25±16.5	715.4±20.5	1.94±1.85	112.7±1.8
Ascorbic acid	100	250.25±19.5	750.5±14.5*	1.56±1.25	119.4±1.5

Table 3 CAT,GSH-Px, SOD activities and MDA level in the liver of mice

Weight (g)	Normal	Aged	*F.carica* leaves Extract 50 mg/kg/day	*F.carica* leaves Extract 150 mg/kg/day	*F.carica* leaves Extract 450 mg/kg/day	Ascorbic acid
Initial body weight	20.5±1.35	22.2±2.12	20.9±1.45	21.5±1.56	20.6±1.5	21.2±1.25
Final body weight	23.9±1.25	30.2±1.75	29.8±2.25	30.9±2.65	27.1±3.21	22.8±1.15
Liver	51.41±0.67	40.47±0.86	44.68±1.45	47.7±0.58	49.94±1.08	52.51±1.47
Kidney	15.60±0.30	11.03±0.64	12.33±0.52	12.98±0.33	14.18±0.32	16.68±0.80
Heart	8.42±0.43	5.16±0.35	6.14±0.35	6.84±0.22	7.1±0.39	9.12±0.57

Table 4 The Effect of *Ficus carica* leaves extract on the body and organ weights of mice

DISCUSSION

Aging is a multifactorial process associated with physiological decline. There is a substantial amount of data supporting the positive relationship between the aging process, and the progressive decline in antioxidant function, combined with increased mitochondrial ROS (reactive oxygen species) generation and accumulation of oxidant products (Song et al., 1999). D-galactose is naturally-occurring, reducing the sugar level in the body and is completely metabolized at a normal concentration. In contrast, at the higher concentrations, it is converted to aldose, hydrogen peroxide, and galactose oxidase; producing superoxide anions and oxygen-derived free radicals that damage macromolecules and cells (Cui et al., 2006). Both enzymatic and nonenzymatic antioxidants exist in intracellular and extracellular environments to quench ROS. The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx) (Ho et al., 2003; Oorojan et al., 2016). From the observation data of Table 2., above, it can be seen that there was an increase in the weight of the mice in all the experimental groups. However, in the negative control group, the mice had a greater increase compared to other groups. This might be due to the continuous induction of D-galactose since D-galactose is a carbohydrate compound belonging to the monosaccharides. The continuous induction of D-galactose for 42 days without any administration of test preparations caused the accumulation of carbohydrates in the body, resulting in increased weight. Galactitol accumulating in the body forms the Advanced Glycation End products (AGEs) through a non-enzymatic process. The greater amount of D-galactose , the more AGEs formed, causing decreased collagen in the skin and greater numbers of free radicals (Ho et al., 2003; Deng et al., 2003). These free radicals attack unsaturated fatty acids, forming a final product of lipid peroxide, i.e., malondialdehyde (MDA), which is toxic in cells.In the aging mice group, antioxidant enzyme activities like CAT, GPx, and SOD are lower than in the treatment group given for *F.carica* and ascorbic acid extracts. The low activities of CAT, GPx, and SOD in the aging group were caused by the induction of D-galactose, which resulted in the autoxidation process of galactose.The autoxidation process of galactose was catalyzed by the small amounts of metal compounds, like iron and zinc. This occurred in phase I of the non-enzymatic glycation process, creating hydrogen peroxide that can inhibit Cu/ZnSOD, which resulted in the

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increased free radical activity and damage caused by the enzyme superoxide dismutase (Oorojan et al., 2016; Cui et al., 2006; Chen et al., 2016).The induction of *F.carica* extract decreased the MDA level and increased antioxidant enzyme activity in all treatment groups with a dosage of 50-450 mg/kg per BW and on the group with ascorbic acid treatment. The research conducted by Caliskan *et al*. (2011) showed that the antioxidant activities of some *F.carica* from Turkey.T he antioxidant capacity of *F.carica* is related to the polyphenols and anthocyanins contained in the fruit. The increased activity of antioxidant enzymes such as CAT, GPx, and SOD is probably due to the fig leaf extract f neutralizing the free radicals, minimizing the formation of AGEs (Caliskan and Polat, 2011; Rahman, 2007) and as a chain-breaking antioxidant by becoming a donor for free radical hydrogen ions so that free radicals became a more stable molecule (Halliwel and Gutterdge,1998). In this way, the free radicals can be neutralized, thus preventing lipid peroxidation in cell membranes. Table 3. shows that the aged groups (only induced by D-galactose with a dosage of 100 mg/kg per BW) with three dosage groups (50 mg/kg of BW, 150 mg/kg of BW and 450 mg/kg of BW) and the ascorbic acid group have a significant difference. This can be interpreted as the effect of the fig leaf ethanol extract dosage of 50-450 mg/kg per BW decreasing the MDA level, although it is not as good as the ascorbic acid positive control. In the aged group, there was a significant increase in MDA level compared to the other groups, due to the induction of D-galactose for 42 days that caused lipid peroxidation. The administration of fig leaf extract with a dosage of 150 mg/kg of BW for 42 days could significantly increase the activity of antioxidant enzymes, such as catalase and glutathione peroxidase, compared to the dosage groups of 50 and 450 mg/kg per BW. At a dosage of 150 mg/kg per BW, the fig leaf ethanol extract could increase the activity of SOD enzyme in the body, proportionally to the ascorbic acid treatment group. This result shows that the higher the dosage of ethanol extract given, the greater the increase in SOD activity. However, at a higher dosage of 450 mg/kg per BW, the SOD enzyme activity decreased. Another parameter used for observing the anti-aging activity of the fig leaf ethanol extract(*Ficus carica* L.) is through the measurement and calculation of mouse organ weight. Table 2. shows that the weight of the organs in the treatment groups with a dosage of 50 mg/kg per BW, 150 mg/kg per BW, and 450 mg/kg per BW had an increase in organ weight compared with the negative control group, (P (0.000) < α). Table 4. also shows that the weight of the organs in the treatment group with a dosage of 50-450 mg/kg per BW increased compared with the negative control group, (P (0.000) < α). The weight profile of the organs presents that the average weight of organs such as the heart, kidneys, and liver in the aging group was lower than in the treatment group (P (0,000) < α). This might be due to the continuous induction of D-galactose. The accumulation of D-galactose can cause aging, and as the aging occurs, the organs in the body experience atrophy, decreasing organ weight. Research conducted by Oroojan stated that the D-galactose-induced mice had a lower weight of their kidneys. D-galactose causes swelling on proximal cells and tubular dilation on the glomerulus (Oorojan et al., 2016). The weights of the livers, heart, and kidneys in the three treatment groups were higher than in the aging group (Table 4). This suggests that the administration of fig leaf ethanol extract protect vital organs. Visually, the three dosage treatment groups showed an increase in the organ index compared to the negative control group. After analysis using the BNT method, it was shown that the treatment group with dosages of 50 and 150 mg/kg per BW was significantly different from the positive control group; however, the treatment group with a dosage of 450 mg/kg per BW had no significant difference. This suggests that the fig leaf ethanol extract with a dosage of 450 mg/kg per BW had the highest anti-aging activity compared to the two dosage treatment groups.The research conducted by Konyaloglu et al. (2005) found that the Turkish fig leaf extract from had a high antioxidant activity, due to the leaves containing α-tocopherol, flavonoid, and phenol. Another study conducted by Rifunschi et al. (2015), also showed the same result from Romanian fig leaves.

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CONCLUSION

This study proves that the fig leaf (*F.carica* Linn) extract causes antioxidant activity and anti-aging in the D-galactose-induced mice. The ongoing research aims to isolate the active compounds of the Indonesian *F.carica* Linn leaves extract so that the antioxidant active compounds can be understood.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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