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Cardioprotective effect of green tea and green coffee extract as metformin's add-on to prevent cardiac fibrosis in a rat model of metabolic syndrome

Indah Nur CHOMSY [1], Mohammad Saifur ROHMAN [2*], Husnul KHOTIMAH [3], Nashi WIDODO [4], Nur Ida Panca NUGRAHINI [5]

1 Doctoral Program in Medical Science, Faculty of Medicine, Universitas Brawijaya, Malang, 65145, Indonesia.
2 Department of Cardiology and Vascular Medicine, Saiful Anwar General Hospital, Faculty of Medicine, Universitas Brawijaya, Malang, 65145, Indonesia.
3 Laboratory of Pharmacology, Faculty of Medicine, Universitas Brawijaya, Malang, 65145, Indonesia
4 Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, East Java, 65145, Indonesia
5 Doctoral Program of Food Sciences, Department of Food Science and Biotechnology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, 65145, Indonesia

* Corresponding Author. E-mail: ippoenk@ub.ac.id (M.S.R)

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ABSTRACT: Metabolic syndrome (METS) is a cluster of risk factors contributing to cardiovascular disease (CVD) development. Agonist-related hypertension and obesity may occur due to excess cardiac pressure caused by increased systemic blood pressure. In addition, METS also enhances the progression of cardiac remodelling through several cardiac fibrosis-related genes. METS treatment is managed using metformin as a glycemic control agent, often given with other complementary agents. This study investigates the effect of green tea and green coffee extract therapy as an add-on to metformin in preventing cardiac organ dysfunction and overexpression of cardiac fibrosis biomarkers in the METS rat model. METS model rats were divided into five groups. The rats' blood pressure, flow, and volume are measured using a non-invasive tail-cuff sphygmomanometer. After nine weeks of treatment, the heart was isolated for measurement of angiotensinogen receptor 1 (ATR1), transforming growth factor β (TGFβ), and collagen type 1 (COL1A1) gene expression by reverse-transcriptase PCR. This study found that there was a significant improvement in blood pressure, increased tail blood flow, and volume (p-value<0.05) and decreased ATR1, TGFβ, and COL1A1 gene expression (p-value=0.000) in the green tea-green coffee and metformin (COMB) therapy group. The correlation analysis results show a positive linear relationship between SBP, DBP, TBF, AT1R, TGFβ, and COL1A1. This study found that green tea and green coffee extract therapy as an add-on to metformin could prevent cardiac dysfunction by improving blood pressure, flow and volume, reducing overexpression of ATR1, TGFβ, COL1A1 gene expression that is related to cardiac fibrosis in the METS rat model.

KEYWORDS: metabolic syndrome; fibrosis; systolic blood pressure; collagen-1; angiotensin receptor 1.

List of abbreviations:
METS: metabolic syndrome
T2DM: type 2 diabetes mellitus
CVD: cardiovascular disease
ATR1: angiotensinogen receptor 1
TGFβ: transforming growth factor β
COL1A1: collagen type 1
EGCG: epigallocatechin-3-gallate
CGA: chlorogenic acid

1. INTRODUCTION

Metabolic syndrome (METS) is a cluster of risk factors for metabolic abnormalities [1]. Risk factors for diagnosing METS include hyperglycemia/insulin resistance, hypertension, obesity, and triglyceride dyslipidemia. These risk factors include a family with a history of coronary disease and smoking habits. According to data from the Centers for Disease Control and Prevention [2], 12.2% of United States adults (18

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years or older) have type 2 diabetes (T2DM). A quarter of these people (23.8%) were unaware they had diabetes. The incidence of T2DM increases with age, reaching 25.2% among the elderly population (65 years and older) worldwide. The prevalence of METS has got three times more than in previous years. Thus, it is estimated that one-third of adults in the United States experience METS. Since 1980, the prevalence of obesity has doubled in 73 countries and is increasing in most of the rest. According to a global survey of obesity incidence in 195 countries, 604 million adults and 108 million children are obese. The most concerning is that the rate of increase is even higher in children [3].

The METS risk factor is an independent factor for the occurrence of cardiovascular disease (CVD). Obesity, hyperglycemia, insulin resistance, dyslipidemia, and increased ROS in response to glucose fluctuations activate inflammatory pathways that are significantly involved in the pathogenesis of cardiac fibrosis. Adipocytes are recognised as a tissue that releases adipokines, leptin, visfatin and cytokines (IL-1, IL-6, TNF-α) that communicate with other tissues through the vascular system distribution [4]. The condition where free fatty acids are elevated, known as dyslipidemia, is known to reduce muscle glucose uptake that exacerbates hyperglycemia [5]. Neurohormonal activation, one of which is the renin-angiotensin-system, also plays a role in the increased risk of CVD in METS. Reactive oxygen species (ROS) are produced when Angiotensin-II activates Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase via the type 1 receptor (ATR1) [6]. Increased ROS in cells triggers other effects, such as increased low-density lipoprotein oxidation and increased translocation of transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), exacerbating inflammation. This condition worsens the work of the heart and stimulates the development of hypertension. Hypertension has an agonist risk with obesity. Hypertension, which occurs because of the increased left ventricular hypertrophy and fibrosis observed in obese individuals, may occur due to excess cardiac pressure caused by increased systemic blood pressure [7].

Fibroblasts are effectors that play an essential role as the primary matrix-producing cells in the cardiac interstitium. Under injury conditions, fibroblasts go through a differentiation process to become myofibroblasts and then express contractile proteins, such as collagen, the main constituent of fibrous fibres [8]. Activation of interstitial fibroblasts in the myocardium in METS sufferers may occur due to significant and persistent metabolic changes. Renin-angiotensin-aldosterone system activation has also been reported in all animal models of obesity and type 2 diabetes, possibly due to metabolic dysregulation, the leading risk factor of METS [9].

Exploration of the beneficial health effects of plants is current research that invites a lot of interest. Plants with polyphenol content that are widely known to have health benefits are green tea and coffee. Epigallocatechin gallate (EGCG) is known to be the most abundant catechin in green tea, representing 50-80% of the total catechins in green tea [10-13]. Consumption of green tea at optimal doses provides many health benefits, such as: preventing the progression of CVD [14,15], regulating blood cholesterol levels [16], increasing fat metabolism, thereby increasing the rate of weight loss [17], slowing cell ageing, reduces inflammation and neurodegenerative diseases [18]. Meanwhile, green coffee is consumed without going through the roasting process (unroasted) [19]. Based on a study by Upadhyay and Mohan [20], chlorogenic acid is the most abundant component in green coffee. Green coffee is considered a source of polyphenol chlorogenic acid (CGA) compared to other sources such as fruits and vegetables [21]. The benefit of CGA has been widely studied and analysed on body weight, fat distribution in the body, and lipid metabolism. One of them was done by Cho et al. [22]. The study showed that CGA increased PPARα expression by increasing fatty acid oxidation in the liver. Hsu et al. [23] reported that CGA inhibited the growth of the preadipocyte cell population. Other studies also report that CGA in coffee can promote a decrease in plasma glucose peaks by weakening intestinal glucose absorption [24,25].

The first-line METS treatment, metformin (1,1-dimethyl biguanide), is a biguanide derivative which works by leading to the activation or phosphorylation of AMP-activated protein kinase (AMPK) [26,27]. Metformin works as a glucose-lowering agent by increasing glucose utilisation, increasing GLP-1, reducing ATP, activating AMPK, increasing insulin sensitivity through effects on fat metabolism, and decreasing cAMP, thereby reducing the expression of gluconeogenic enzymes [28]. METS treatment, which has so far focused on individual components by suppressing insulin resistance using metformin, can be improved with other complementary agents [29]. Complementary agents enhance the effect of a single treatment, which is expected to prevent the worsening of progression and complications caused to the heart. However, not many studies investigated the complementary effects of herbs on their use with drugs in the treatment of METS. Therefore, this study aimed to investigate the effect of green tea and green coffee extract therapy as an add-on to metformin in preventing cardiac organ dysfunction and overexpression of cardiac fibrosis biomarkers in the METS rat model.

2. RESULTS

2.1. Improved blood pressure, increased tail blood flow, and volume in the green tea-green coffee and metformin combination therapy group

This study found that combination therapy (COMB) gave good results on blood pressure, tail blood flow, and blood volume, which can be seen in Table 1 and Figure 1. Systolic blood pressure measurements (SBP, mmHg) showed a decrease in the therapy group, alone or in combination. In the NORM group, the average SBP was 93.80 ± 1.85, while the METS group had the highest average SBP, 186.60 ± 15.93 mmHg. In the MFN group, it was found that SBP was 137.80 ± 18.49, GTCE was 169.80 ± 2.26, and COMB had the lowest SBP among the treatment groups, 116.00 ± 6.47. These five groups differ significantly with p-value = 0.000. On diastolic blood pressure (DBP) measurements, in the NORM group, the average DBP was 88.00 ± 14.66, and the highest in the METS group was 173.40 ± 13.38. In the MFN group, there was a decrease in DBP to 116.80 ± 28.32, as well as in the GTCE group, which had a DBP of 152.20 ± 6.82. The most significant reduction in DBP occurred in the COMB 87.40 ± 3.35 group. DBP is significantly different with p-value = 0.003.

Table 1. Results of measurement and analysis on SBP, DBP, TBF, and BV

Variable	Group (n=5)	Min	Max	Mean Square^	F	p-value
Systolic Blood Pressure (SBP)	NORM	90.00	100.00	7214.60	11.155	0.000*
	METS	142.00	230.00			
	MFN	100.00	184.00			
	GTCE	161.00	174.00			
	COMB	102.00	140.00			
Diastolic Blood Pressure (DBP)	NORM	58.00	141.00	7402.54	5.903	0.003*
	METS	133.00	200.00			
	MFN	120.00	184.00			
	GTCE	135.00	172.00			
	COMB	79.00	99.00			
Tail Blood Flow (TBF)	NORM	4.77	9.71	121.47	10.969	0.000*
	METS	10.62	25.09			
	MFN	2.03	8.59			
	GTCE	3.53	8.90			
	COMB	3.45	6.17			
Blood Volume (BV)	NORM	3.98	11.71	27.96	2.033	0.128
	METS	0.30	8.62			
	MFN	0.05	13.41			
	GTCE	1.05	9.24			
	COMB	3.47	8.91			

^ Mean square in between group
* Significant different (p-value< 0.05)

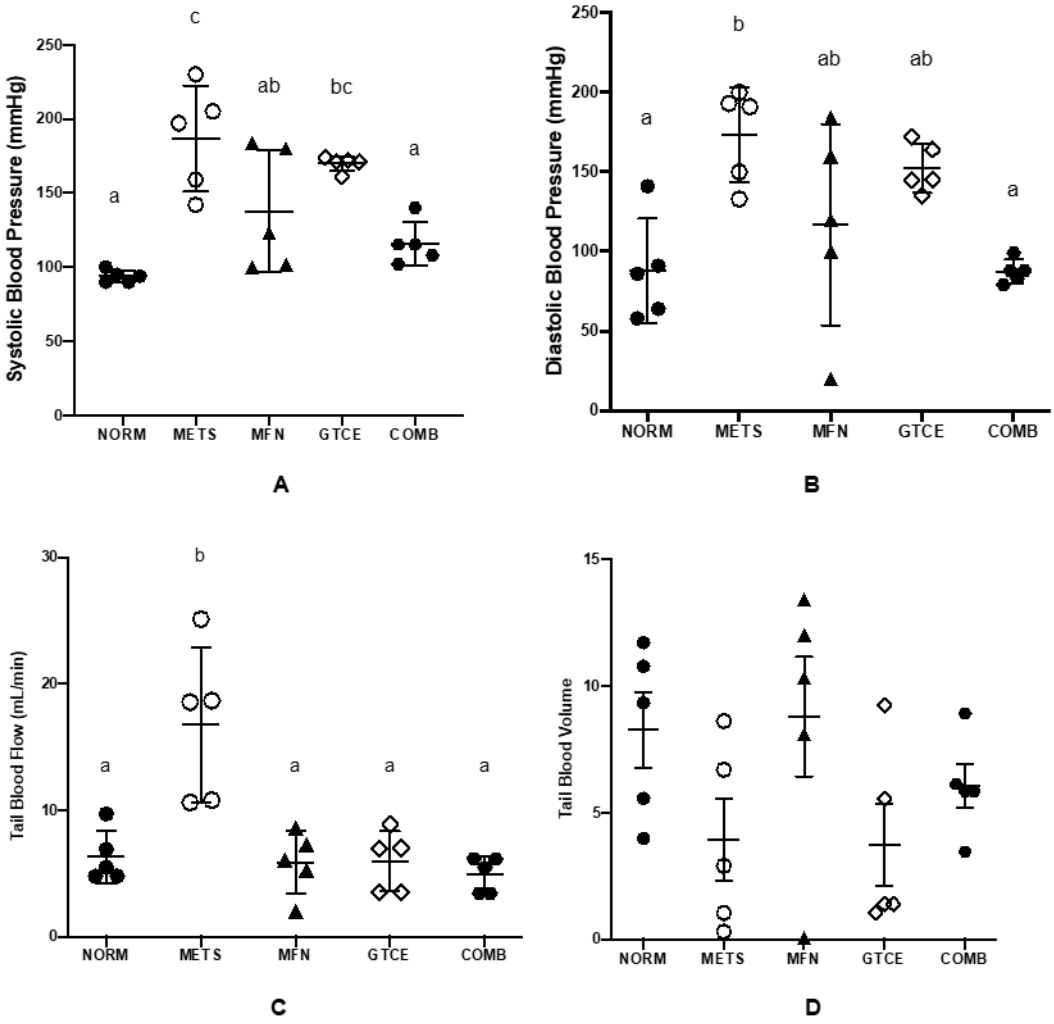

Figure 1. Measurement of SBP, DBP, TBF, BV and findings in this study. (A): Systolic Blood Pressure, (B): Diastolic Blood Pressure, (C): Tail Blood Flow, and (D): Tail Blood Volume results in all groups. Data are expressed as mean ± SEM or SD (n=5). The groups which did not share the same letters in the same row were significantly different by ANOVA followed by a Tukey's test ($p<0.05$).

Tail blood flow (TBF) observed in this study also showed improvement in each treatment group. TBF in the NORM group had the lowest value, 6.34 ± 0.93, while the highest was in the METS group, 16.74 ± 2.73. Blood flow in the MFN group was 5.87 ± 1.11, while in the GTCE group, it was 5.99 ± 1.06. The COMB group had the lowest blood flow, 4.93 ± 0.62. Based on the one-way ANOVA analysis, the five groups on the TBF measurement differed significantly with a p-value = 0.000. Blood volume measurements using the tail-cuff sphygmomanometer showed that the NORM and MFN groups had nearly the same average values, 8.27 ± 1.50 and 8.78 ± 2.36, respectively. However, there was a decrease in the METS and GTCE groups up to 3.91 ± 1.62 and 3.72 ± 1.61. In the COMB group, it was found that the lowest average blood volume was 6.04 ± 0.86. However, on this blood volume measurement, the five groups did not differ significantly ($p>0.05$).

2.2. Decreased the Angiotensinogen Receptor 1 (ATR1), Transforming Growth Factor β (TGFβ), and Collagen Type 1 (COL1A1) gene expression due to green tea-green coffee and metformin therapy

This study found that combination therapy (COMB) gave good results on target gene expression. The measurement and analysis results can be seen in Table 2 and Figure 3. Gene expression in this study is the expression of the target gene relative to the expression of its housekeeping gene, namely β-actin. In measuring the expression of the ATR1 gene, it can be seen that the NORM group as a control has the lowest relative expression of 0.246 ± 0.037. Meanwhile, the highest expression in METS was 0.723 ± 0.048. The relative expression of the ATR1 gene is known to decrease in the group given the therapy. Single therapy from the

MFN and GTCE groups gave gene expression of 0.404 ± 0.019 and 0.433 ± 0.051. However, the COMB therapy group was known to have the lowest gene expression compared to all single therapy groups, 0.383 ± 0.031.

Table 2. Results of measurement and analysis on gene expression ATR1, TGFβ, and COL1A1

Variable	Group (n=5)	Min	Max	Mean Square^	F	p-value
ATR1	NORM	0.13	0.34	0.153	19.912	0.000*
	METS	0.65	0.91			
	MFN	0.35	0.46			
	GTCE	0.25	0.56			
	COMB	0.29	0.45			
TGFβ	NORM	0.16	0.60	1.792	39.7	0.000*
	METS	1.50	2.15			
	MFN	0.54	1.23			
	GTCE	0.38	0.68			
	COMB	0.16	0.58			
COL1A1	NORM	0.16	0.36	2.540	142.19	0.000*
	METS	1.87	2.39			
	MFN	0.64	0.97			
	GTCE	0.82	0.95			
	COMB	0.26	0.56			

^ Mean square in between group
* Significant different (p-value< 0.05)

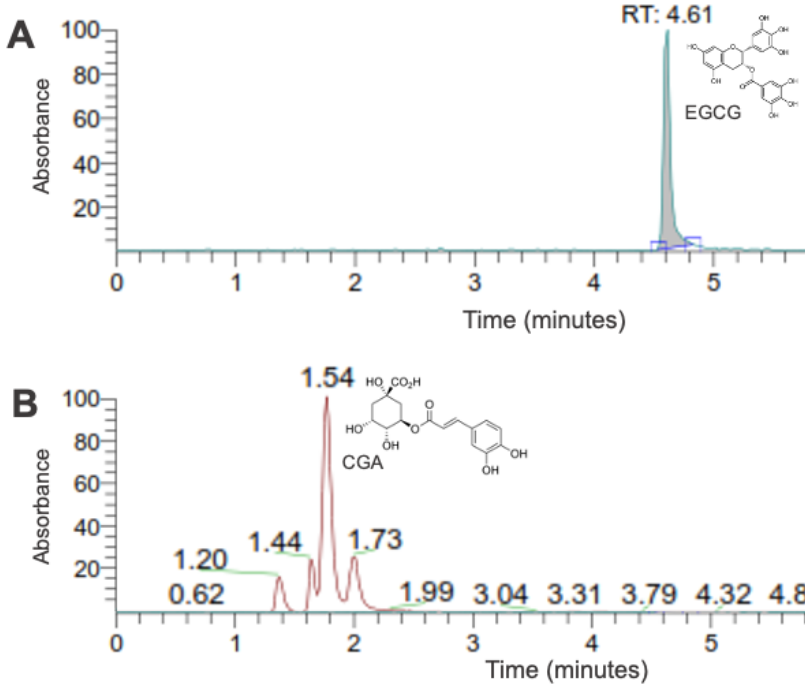

Figure 2. High-performance liquid chromatography (HPLC) profile of extract: (A) green tea and (B) green coffee.
Note: RT: retention time; EGCG: epigallocatechin-3-gallate; CGA: chlorogenic acid

Measurement of TGFβ and COL1A1 gene expression, both gene expressions were found to be highest in the METS group, 1.770 ± 0.107 for TGFβ and 2.061 ± 0.095 for COL1A1, and lowest in the NORM group, 0.359 ± 0.079 for TGFβ and 0.241 ± 0.034 for COL1A1. This expression then decreased in the MFN (0.990 ± 0.134 and 0.830 ± 0.059) and GTCE (0.521 ± 0.051 and 0.892 ± 0.024) groups. Meanwhile, the lowest gene expression

in both genes was again found to be lowest in the COMB therapy group, 0.372 ± 0.082 and 0.405 ± 0.061. All groups in measuring the expression of the ATR1, TGFβ, and COL1A1 genes in this study were significantly different with a p-value = 0.000 (Figure 2).

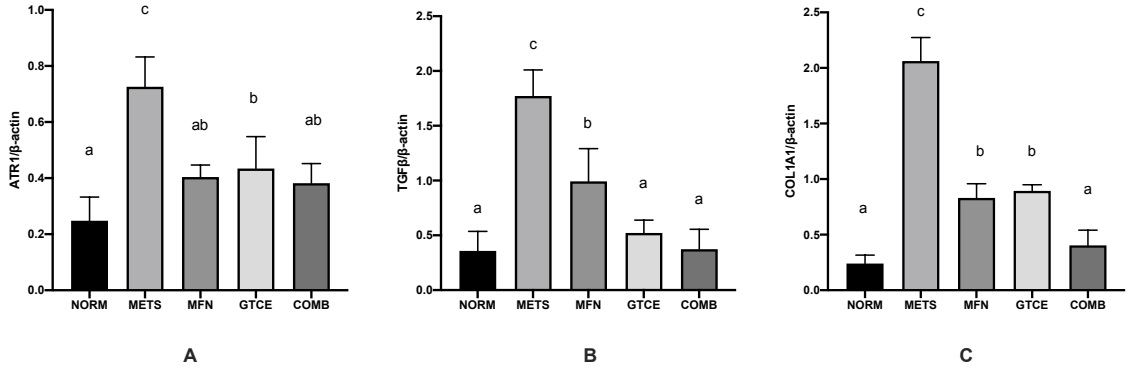

Figure 3. Measurement of the gene expression of ATR1, TGFβ, and COL1A1 and findings in this study. (A): Angiotensin Receptor 1, (B): Transforming Growth Factor-β, and (C): Collagen type 1 results in all groups. Data are expressed as mean ± SEM or SD (n=5). The groups that did not share the same letters in the row were significantly different by ANOVA followed by a Tukey's test ($p<0.05$).

2.3. Correlation between SBP, DBP, TBF, and BV with ATR1, TGFβ, and COL1A1 gene expression

This study found a correlation between the parameters measured in this study. The correlation between the variables in this study illustrates the relationship related to one another. The correlation between variables can be seen in Table 3. Based on the results of the analysis, it is known that several variables are positively correlated. SBP, DBP, TBF, ATR1, TGFβ, and COL1A1 were eligible for analysis using Pearson's correlation. The reason was that the data was homogeneous and normally distributed. Meanwhile, BV data was analysed using Spearman's correlation because the data was not normally distributed. The analysis results show a linear and positive relationship between SBP, DBP, TBF and ATR1, TGFβ, and COL1A1. The variable SBP demonstrated the strongest correlation with cardiac fibrosis-related genes. A strong correlation was also shown in the analysis results of TBF with COL1A1. These results showed that the changes in SBP, DBP, and TBF are also related to the ATR1, TGFβ, and COL1A1 gene expression. Correlation in a negative direction/reverse was found in BV, with the highest value in the correlation with the COL1A1 gene. In this condition, the relationship reflected by BV with gene expression is reversed. For example, a decrease in the BV value will be followed by an increase in the ATR1, TGFβ, and COL1A1 gene expression, and vice versa.

Table 3. Results of correlation analysis

Variable	ATR1	TGFβ	COL1A1
SBP	0.675**	0.615**	0.742**
DBP	0.552**	0.623**	0.681**
TBF	0.654**	0.668**	0.786**
BV	-0.201	-0.293	-0.365

**Correlation is significant at the 0.01 level (2-tailed).

3. DISCUSSION

METS is an accumulation of several risk factors that adversely affect body systems. The accumulation of risk factors in METS sufferers is known to cause many organ complications that even lead to death. Obesity, dyslipidemia, hypertension, glucose intolerance/insulin resistance, and other risk factors put people at higher risk of developing heart disease [30-33]. Metabolic disorder accompanied by an increase in the heart's workload is then known to be the trigger for changes in the heart's structure. The heart's structural changes in METS can directly affect increased adiposity or reflect the consequences of multiple pathophysiologies.

Hypertension in obese individuals occurs due to increased left ventricular hypertrophy, and fibrosis can occur due to excess cardiac pressure caused by increased systemic blood pressure [34,35]. Excessive increases in pressure and volume can trigger concentric hypertrophy associated with increased collagen deposition and diastolic dysfunction, while excessive volume causes dilation accompanied by matrix degradation [7]. The relative correlation of pathophysiological changes in increased pressure and volume in the remodelling process in obese subjects is still uncertain. Meanwhile, fasting hyperglycemia, insulin resistance and triglyceride dyslipidemia, which are very common in obese patients, are believed to be involved in remodelling cardiac fibrosis and hypertrophy. Increased oxidative stress in response to glucose fluctuations activates inflammatory pathways that are significantly involved in the pathogenesis of fibrosis and cardiac remodelling [36,37].

In this study, it was found that in the METS group, there was an increase in blood pressure, both systolic and diastolic, as well as significant changes in blood flow and volume (p-value <0.05). Hypertension in the METS group is early evidence that there has been compensation for the increased heart workload due to METS. Even so, it is known that there are improvements in these four parameters in the administration of either single therapy with MFN or GTCE alone. In treatment using a combination of herbal drugs, such as in the COMB group, it was found that the most significant decrease was found among the groups given single therapy. This finding becomes interesting because of the complementary effect of the combination of green tea and green coffee as an add-on to metformin in lowering blood pressure.

It is well known that the relationship between TGF-β and cardiac fibrosis has been well established [38, 39]. TGF-β fibrogenic activity may occur mediated through the transcription factor Smad signalling [39], although independent activation pathways (without Smad activation, non-canonical) can also be directly involved. Increased myocardial TGF-β expression was found consistently in obesity models and is closely associated with cardiac fibrosis [40,41]. The upregulation of TGF-β in obesity-associated cardiomyopathy may be due to the activation of AT1 by angiotensin II. Still, it may also be due to activating an angiotensin-independent pathway through stimulating high glucose and leptin levels in the TGFβ transcription and activation process. TGF-β exerts an intense pro-hypertrophic action and promotes matrix production in cardiac fibroblasts [39]. In addition to finding improvements in blood pressure, the green tea-green coffee combination with metformin also decreased the expression of genes related to cardiac fibrosis. The ATR1, TGFβ, and COL1A1 gene expression were significantly reduced in the COMB-treated group (p-value=0.000). These results also show that the activity of polyphenols from green tea and green coffee, which work together with metformin, can modulate metabolism at the gene expression level, thereby preventing the progression of cardiac remodelling.

The content of green tea polyphenols, EGCG, is known to have many health benefits. In research conducted by Zhong et al. using a high-fat diet rat model, administration of EGCG improved tissue abnormalities and morphology of myocardial tissue through activation of Sirtuin-1, endothelial nitric oxide synthase (eNOS) and AMP-activated protein kinase α through stimulation of lipid metabolism [42]. Other studies support this finding by stating that EGCG protects TAC model mice by regulating the Akt/mTOR pathway [43]. EGCG also reduces cardiac hypertrophy caused by angiotensin by inhibiting the NFkB signal pathway [44]. Meanwhile, CGA, as the main ingredient of green coffee, is also known to have a positive effect on reducing systolic blood pressure and heart rate in hypertensive rats by cyclosporine induction [45] while restoring the activity of acetylcholinesterase, butyrylcholinesterase, and arginase which changed in hypercholesterolemic rats [46]. The cardioprotective effect of consumption of green tea-green coffee was also found in previous studies, decreased expression of the fibrotic galectin-3 gene in the METS model [47]. However, in this study, the control over the fibrotic genes given by MFN increased with the consumption of green tea-green coffee. This condition shows that green tea and green coffee have a good cardioprotective effect as complementary agents to the first line of METS therapy, metformin. The escalation of the therapeutic effect of using these two materials provides good insight into non-invasive treatment in treating metabolic syndrome. Further research is needed to elucidate the mechanism of a specific pathway in inhibiting the progression of cardiac remodelling in metabolic syndrome.

4. CONCLUSION

This study found that green tea and green coffee extract therapy as an add-on to metformin could prevent cardiac dysfunction by improving blood pressure, flow and volume, reducing overexpression of angiotensin receptor 1, transforming growth factor β, collagen type 1 gene expression that is related to cardiac fibrosis in the METS rat model.

However, this study has several limitations. The number of samples and the period of this investigation are both constrained. However, before it can be used in people, the mixture of green tea, green coffee, and metformin has to be examined on a more significant number of samples for a more extended period. Measuring the protein linked to the downstream end of the pathway we employed in this investigation is necessary for further exploration.

5. MATERIALS AND METHODS

5.1 Animals

Rats (*Rattus norvegicus*) were used in this work as experimental animals under the replacement, reduction, and refinement (3R) principles. The process was under the rigorous supervision of the ethical committee to adhere to the institution's rules for using experimental animals. The number of rats in this study was kept to a minimum, and each treatment given to experimental rats was less painful. The initial seven days after arriving were spent acclimatising. A polycarbonate cage (90 x 60 x 60 cm) with hemp bedding was used to house each rat. At a temperature of 25°C, 50% humidity, and 12-hour light/dark cycles, the environment was maintenance-stable. Food was given out and restocked every day. *Ad libitum* access to the beverage was available.

5.2 Research Design

After a week of acclimatisation, male Sprague-Dawley rats (4 weeks old) weighing 150 grams were randomly divided into five groups (n = 5). A diet high in fat and high in sugar (HFS) was given to the rats to induce obesity and high blood sugar as the METS eligibility model. The rats were injected with low doses of STZ (30 mg/kg body weight diluted with 10% citrate buffer pH 4.5) intraperitoneally when they were 10-11 weeks old and weighed between 480- 500 grams. The rat needs to meet the following three primary NCEP-ATP III 2005 recommendations to qualify as a METS model: high fasting blood glucose (>200 mg/dL), high triglycerides (TG>200 mg/dL), and high-density lipoprotein (HDL40 mg/dL). For 4-6 weeks, all risk factors must remain stable [48]. The rat model of METS was then divided into three treatment groups: one that received metformin (MFN, 100 mg/kg BW), one that received green tea and green coffee extracts (GTCE, green tea extracts 300 mg/kg BW, and green coffee extracts 200 mg/kg BW), and group who received a combination of metformin and the green tea-green coffee extracts (COMB, metformin 100 mg/kg BW, green tea extracts 300 mg/kg BW and green coffee extracts 200 mg/kg BW). Furthermore, we employ the negative control (NORM) and the positive control groups (METS). The extract and metformin dosage results from a preliminary investigation to identify the ideal dose that yields a significant result and is known to be safe [49]. Oral gavage provides the medicinal component after being dissolved in mineral water. The therapy was carried out for nine weeks, administered once a day, and given in the afternoon. Each element of treatment was provided according to the group and the dose. In this study, researchers measured daily food consumption and fluid given *ad libitum*.

5.3 Plant Material

The green tea leaf utilised in this study was grade 1 quality, the top new leaf, and leaf buds. All the leaves were purchased from Ciwidey, Indonesia (7°9'24.48''S-108°0'23.4''E). Dried green tea and coffee beans were sorted to remove contaminants or low-quality green tea after harvest. Meanwhile, the green coffee bean in this study was a premium coffee bean purchased from Dampit, Indonesia (8°44'16.64''S-113°41'52.26''E). After confirmation from farmers and distributors, the laboratory assistant carried out the identification and quality checking of the variants. The sample was deposited as an example in the Molecular Biology Laboratory at Universitas Brawijaya with the reference number TSN-506801 for green tea and KAD-001 for green coffee beans.

5.4 Extraction of green tea and green coffee

Sorted green tea leaves and green coffee beans are roasted in an oven at 180°C for three minutes for green tea leaves or until the first crack appears for the coffee bean. In general, this study used conventional extraction methods, which are agitation methods with slight modifications in temperature and time. Demineralised drinking water was used as the solvent for the extraction, with a sample-to-solvent ratio of 1:15, and it was done at 90°C for 10, 20, and 30 minutes. The coarse filter paper was then used to filter the sample. Green coffee extract is decaffeinated using activated carbon. Then, 2.5% w/v water, 0.5% w/v water formic acid, and 25% w/v water-activated carbon were added with cane sugar. The mixture was incubated for 6 hours at 80°C in a water bath shaker. Then, the activated carbon (200% v/w activated carbon) was removed from the solvent and washed. The decaffeination of green coffee extract was accomplished using activated carbon at a ratio of activated carbon to green coffee extract of 1:75 (w/v extract). A water bath shaker was used to conduct the decaffeination process at 60°C, 70°C, and 80°C for 6, 7, and 8 hours before filtering. The blanching procedure was modified to decaffeinate green tea [50]. The decaffeinated process for green tea was done at 50, 75, and 100°C for 1, 3, and 5 minutes, respectively. Green tea and decaffeinated coffee extracts were combined with 5% maltodextrin (w/v extract). It was then dried for five hours at 60°C in a food dehydrator. A size reduction was accomplished using a dry blender and an 80-mesh sieve. Using high-performance liquid chromatography (HPLC) equipment, the EGCG content of green tea extract and the CGA portion of green coffee extract were examined (Shimadzu Corporation, Japan). According to HPLC measurement, the highest content in the green coffee extract is CGA fraction, and in green tea extract is EGCG (Figure 3).

5.5 Measurement of Blood Pressure, Flow, and Volume Pressure

Measurements were conducted using the Model CODA® Standart, a non-invasive tail-cuff method (Kent Scientific, Torrington, USA). Rats are placed in appropriate holders based on their size. Rats were picked up by the tail and gently placed into the rear of the holder with faces at the open end of the nose cone. The rear hatch is carefully secured by turning a screw on the rear hatch to ensure that no other body parts are caught. The nose cone shifted toward the rear hatch to limit animal movement. The holder occupied by the rat is placed onto the warming platform in the designated position. The animal will be acclimated for at least 5 min to the holder (32-35°C) without any unnecessary contact that could irritate the animal. The occlusion tail-cuff is placed through the tail and to the base of the tail without force. After that, the tail was inserted through the volüme pressure recording (VPR) sensor cuff and placed within 2 mm of the occlusion cuff. Cycles were carried out for ten cycles for each rat as the final measurement.

5.6 Measurement of Angiotensinogen Receptor 1 (ATR1), Transforming Growth Factor β (TGFβ), and Collagen Type 1 (COL1A1) gene expression

Fresh heart tissue was taken in 3 grams, mashed with a sterile mortar and pestle, and 500 µL of PrimeZol was added gradually. 300 µL of chloroform was added, vortexed for 10 seconds, and then centrifuged at 13,000 RPM, 4°C, for 15 minutes. A 500 µL isopropanol was added to a new tube. The supernatant was taken and put in an isopropanol tube (1:1). 3M potassium acetate was added 30 µL to each sample, then mixed well. Incubation was carried out overnight at -20°C. After that, the samples were centrifuged at 13,000 RPM, 4oC, for 10 minutes, and the supernatant was discarded. Pellets were added with 75% ethanol 1000 µL, mixed until the pellets floated in ethanol. Samples were centrifuged again at 13,000 RPM, 4°C, for 5 minutes. The supernatant was discarded, the pellet was dried in LAF for 1 hour, and 30 µL of RNAse-free water was added. Heart RNA extract concentration was measured using Nanodrop and made into cDNA via reverse-transcriptase PCR. RT reactions were done using a ReverTra Ace kit (Toyobo, Japan). The RNA expression level was determined using the LightCycler 96 PCR system (Takara, Japan) and the GoTaq Green Master PCR Kit (Promega, Madison, United States) according to the manufacturer's instructions. The sequence of the primers is as follows: β-actin, forward: 5'- TGA GAG GGA AAT CGT GCG TGA CAT-3' and reverse: 5'-ACC GCT CAT TGC CGA TAG TGA TGA-3'; AT1R forward 5'-TGC CAT GCC CAT AAC CAT CTG-3', reverse 5'-CGT GCT CAT TTT CGT AGA CAG G-3; TGFβ1, forward: 5'- TCC TGT CCA AAC TAA GGC TC-3'; reverse: 5'- CAA GGT AAC GCC AGG AAT TG-3'; and COL1A1, forward: forward: 5'-CTG GGC CTA TCT GAT GAT CT-3' and reverse: 5'- TGC TTT GGA AAA TGG TGC TCT G-3'. The cycle of PCR was as follows: 5 minutes at 95 °C for predenaturation; 29 cycles of 30 seconds of denaturation at 95 °C, 30 seconds of annealing at 55 °C, and 30 seconds of extension at 72 °C; and a 10-minute extension at 72 °C. The mRNA

expression level of the target gene was normalised to the expression level of β-actin. The results were analysed with ImageJ software.

5.7 Statistical analysis

Statistical analysis was performed using SPSS 20.0 and GraphPad Prism 9.0 software. Data are presented as mean ± standard deviation. Test for data normality and homogeneity using the Kolmogorov-Smirnov/Shapiro-Wilk test and Levene's test ($p > 0.05$). Statistical tests were carried out using Duncan's ANOVA-Post Hoc variation test. Correlation analysis was performed using the Pearson and Spearman tests. P-values less than 0.05 were considered significant.

5.8 Ethical Clearance

This experimental design has been fulfilled and approved by the Health Research Ethics Committee of Saiful Anwar General Hospital, Malang, Indonesia, by registered number: 400/211/K.3/302/2021.

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