Supplementation Of Caesalpinia Sappan L. Extract to Suppress Caspase 3 In Sprague Dawley Rats Exposed To Formaldehyde Gas By Inhalation

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Abstract

Many types of occupational activities determine formaldehyde (FA) exposure. FA inhaled causes oxidative stress, activates Caspase-3, -6, and -7, and causes apoptosis in nasal epithelial cells. Inhalation of FA has also been shown to cause cell proliferation and tissue damage. Free radicals and oxidative damage can be prevented and inhibited by antioxidant compounds, one of which is Sappan wood (*Caesalpinia sappan L*). There has been no recent review analyzing the use of *Caesalpinia sappan L* in suppressing the incidence of apoptosis due to exposure to inhaled FA. This research is true experimental research in vivo with a randomized post-test-only control group design. The subjects of the study were 30 male *Sprague Dawley* rats, divided into 6 groups. FA was presented for 8 hours/day following the daily pattern of labor hours for 2 weeks. Rats are given Sappan wood extract for 28 days. Blood serum was analyzed to measure apoptosis through caspase 3 (measured by the ELISA method). Data were analyzed using Kruskall Wallis and Mann-Whitney test with a 95% meaningfulness level. Caspase 3 ranges between 0.13404 - 0.13406 pg/ml. The best levels of caspase 3 are in the sappan wood extract (SWE) 1000 group and positive control. There were significant differences in caspase 3 levels. A dose of Sappan wood extract 1000 mg/kg body weight/day produced the best levels of caspase 3 among all groups.

Keywords: supplementation, *Caesalpinia sappan l.* extract, caspase 3, rats, exposed to formaldehyde gas

DOI: 10.48047/ecb/2023.12.8.735

INTRODUCTION

Formaldehyde (FA) is one of the chemicals that are widely used in industry today. FA is widely applied in many production processes, such as the construction materials industry, the chemical industry (resins, paintings, etc.), the wood-processing and furniture industry, the food industry, biomedical laboratories, gross anatomy rooms, handicrafts, etc [1]. Consequently, many types of occupational activities determine FA exposure. Exposure occurs primarily by inhaling airborne FA, but it can also be absorbed through the skin or ingested. Acute and chronic inhaled formaldehyde has been linked to a variety of toxic effects on the liver, nerves, reproductive system, respiratory tract, and cancer in numerous epidemiological and animal studies [2]–[4]. The *National*

Academy of Science (NAS) dan the *International Agency for Research on Cancer* (IARC) concluded that formaldehyde interacts with DNA, RNA, and proteins at the site of first contact causing carcinogenic effects [4],[5].

DNA damage by free radicals causes carcinogenesis in several ways, namely changing the structure of DNA, affecting cytoplasmic and nuclear signal transduction pathways, and modulating the activity of proteins and genes that contribute to oxidative stress and regulation of cell proliferation, differentiation, and apoptosis [6],[7],[8]. DNA changes are responded to by activating the P53 protein (tumor suppressor protein). Then P53 will modulate the expression of proteins that control mitochondrial membrane permeability and cause the release of mitochondrial proteins such as cytochrome C. Furthermore cytochrome C and Apoptotic Protease Activating Factor 1 (Apaf-1) form apoptosomes that activate Caspase-9 (caspase initiator of apoptosis). Then the executor of caspase regulates the dismantling of cell structures, disrupts cell metabolism, inactivates cell death-inhibiting proteins, and activates damaging enzymes, so that DNA damaged was destroyed so as not to cause continued cell abnormalities [9]. Formaldehyde (40 mL, 400 mM) inhaled by male F344 rats can activate Caspase-3, -6, and -7 through the induction of FasL (Fas ligand) and TNFR (*tumor necrosis factor receptor*) in nasal epithelial cells [10].

Free radicals and oxidative damage can be prevented and inhibited by antioxidant compounds, by adding hydrogen atomic groups to electrons that do not have pairs so that they are stable [12], [13]. One antioxidant that is relatively easy to obtain and affordable is Sappan wood (*Caesalpinia sappan L*). Phytochemically Sappan wood contains alkaloids, flavonoids, saponins, tannins, and terpenoids. Brazilein ($C_6H_{14}O_5$) is included in the flavonoid group which can inhibit the formation of hydroxyl free radicals, superoxide anions, peroxyl, alkoxyl, singlet oxygen, hydrogen peroxide, and inhibit apoptotic inhibitor proteins [14], [15], [16], and engaged in the activation of Caspase 3 and Caspase 9 [17]. Strong antioxidants from nature such as Sappan wood are expected to replace synthetic antioxidants because they have a better level of cell safety. Some previous reviews have investigated the relationship between occupational formaldehyde exposure and the onset of certain cancers, but have often reached conflicting conclusions [12], [13], [18] There has been no recent review analyzing the use of *Caesalpinia sappan L* in suppressing the incidence of apoptosis due to exposure to inhaled formaldehyde.

Method:

This research is true experimental research in vivo and use randomized post test only control group design [19]. The subjects were male *Sprague Dawley* rats, 2-4 months old, with body weight 200-300g, and in healthy condition (active, not disabled). Calculation of the number of samples with the Federer formula. The number of treatment groups was 6, the number of replications was 5, so the total number of subjects was 30.

Sappan Wood Extraction (SWE)

175 grams of Sappan wood simplisia added 80% ethanol until submerged, then stirred for 1 hour and allowed to stand 24 hours. Next it is filtered and separated pulp and filtrate. In the pulp added 80% ethanol and stirred again 1 hour, and allowed to stand 24 hours (Maceration was carried out 3 times). After the whole filtrate is collected from 3 extractions, it is then concentrated with a *rotary evaporator* with a temperature of 60 ° C then evaporated on a waterbath until a constant weight is obtained [20].

Formaldehyde Gas Exposure and Sappan Wood Extract Administration

Before treatment, rats were adapted for 7 days in cages in animal laboratories and given standard feed as much as 10% body weight/day and drinks. Each treatment's experimental cage was made of 2 plastic boxes. In the treatment, each rat needs 2 plastic boxes. The first box is made of a hole for the circulation of breathing air, this is where the rats were placed. In the second box was placed a glass containing 10% liquid formaldehyde that is allowed to evaporate [21]. The first box is connected to the second box through a plastic pipe. Formaldehyde vapors from the second box will enter the first box for rats to inhale [21].

After 7 day of adaptation, group 1 rats were only given standard food for 14 days [22]. Group 2 was exposed to formaldehyde for 10 days, group 3 was exposed to formaldehyde for 10 days and SWE for 14 days, and group 4 was given SWE for 14 days [23], then given formaldehyde and SWE again for 10 days. Formaldehyde was presented for 8 hours/day following the daily pattern of labor hours [24]. The complete trial design is as follows:

Negative control group	:	Formaldehyde gas exposure for 5 days, then 2 days without exposure, followed by 5 days exposure, and another 2 days without exposure
Positive control group	:	Rats are given vitamin C for 14 days. Then on day 15 exposure to formaldehyde gas was given for 5 days, then 2 days without exposure, followed by exposure for 5 days, and 2 days without exposure. During formaldehyde exposure remains given vitamin C
Normal control group	:	Rats are given standard feed for 28 days
SWE 1000 Group	:	Rats are given SWE 1000 mg/kg body weight for 14 days. Then on day 15 exposure to formaldehyde gas was given for 5 days, then 2 days without exposure, followed by exposure for 5 days, and 2 days without exposure. During formaldehyde exposure remains given SWE 1000 mg/kg body weight
SWE 400 Group	:	Rats are given SWE 400 mg/kg body weight for 14 days. Then on day 15 exposure to formaldehyde gas was given for 5 days, then 2 days without exposure, followed by exposure for 5 days, and 2 days without exposure. During formaldehyde exposure remains given SWE 400 mg/kg body weight
SWE Group 100	:	Rats are given SWE 100 mg/kg body weight for 14 days. Then on day 15 exposure to formaldehyde gas was given for 5 days, then 2 days without exposure, followed by exposure for 5 days, and 2 days without exposure. During formaldehyde exposure remains given SWE 100 mg/kg body weight

* SWE= Sappan wood extract

After the intervention, the rats were anesthetized with ketamine 0.2 mg/kg body weight and xylazine 0.1 mg/kg body weight orally, turned off by dislocation of the neck, dissected and blood

drawn through the heart. Furthermore, blood serum was analyzed to measure cell damage through caspase 3 measurements. Caspase 3 was measured by the ELISA method [25]. Data analysis was carried out descriptively and analytically with the Kruskall Wallis and Mann-Whitney test with a 95% meaningfulness level. Ethical clearance was obtained from the Komite Etik Fakultas Kedokteran Universitas Diponegoro.

RESULTS

1. Caspase 3 Levels In Among Groups

The distribution of Caspase 3 levels by treatment group is listed in table 1 as follows:

Table 1. Caspase 3 (pg/ml) Blood Serum Levels of Rats in Various Treatments

Treatment groups	Minimum	Maximum	Average
Normal control	0.13404	0.13406	0.134050 (SD±7.07107E-06)
Negative control	0.13404	0.13408	0.134058 (SD±1.64317E-05)
Positive control	0.13404	0.13404	0.134040 (SD±0)
SWE 1000	0.13404	0.13404	0.134040 (SD±0)
SWE 400	0.13404	0.13405	0.134042 (SD±4.47214E-06)
SWE 100	0.13404	0.13406	0.134044 (SD±8.94427E-06)

*SWE= Sappan wood extract

Based on Table 1, the best levels of caspase 3 were in the positive control group and SWE 1000, so it can be concluded that vitamin C and SWE dose 1000 provided the best effect in suppressing apoptotic. When compared to the normal control group, all groups that received SWE had better levels of caspase 3. The worst levels of caspase 3 were in the negative control group. There were

significant differences in caspase 3 levels among all groups (p Kruskal Wallis = 0.009). Analysis of caspase 3 between pairs of treatment groups, were in Table 2 below:

Table 2. Differences In Caspase 3 Levels In Various Treatments

Treatment Group	p- value Caspase 3
Normal control- Negative control	0.222
Normal control - Positive control	**0.032**
Normal control – SWE 1000	**0.032**
Normal control - SWE 400	0.095
Normal control - SWE 100	0.222
Negative control- Positive control	**0.032**
Negative control– SWE 1000	**0.032**
Negative control– SWE 400	0.056
Negative control– SWE 100	0.151
Positive control - SWE 1000	1.000
Positive control - SWE 400	0.690
Positive control - SWE 100	0.690
SWE 1000 - SWE 400	0.690
SWE 1000 - SWE 100	0.690
SWE 400 - SWE 100	1.000

When compared to normal controls, only positive controls and SWE 1000 had significantly lower caspase 3 levels. This also occurred when compared to the negative control group. The levels of caspase 3 in SWE 1000 groups were the same as those who got vitamin C. This strengthens the conclusion that the ability of SWE dose 1000 is comparable to vitamin C in suppressing the formation of caspase 3.

2. Caspase 3 Levels Based on The Dose of Sappan Wood Extract

Descriptive analysis of caspase 3 levels at various doses of Sappan wood extract when compared with a negative control group is shown in figure 2 below:

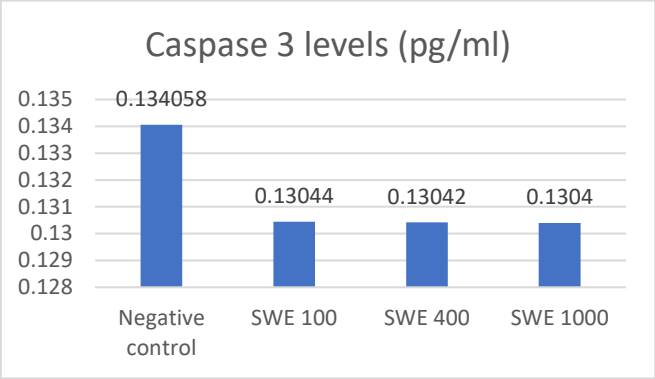

Figure 2. Average of Caspase 3 levels based on the dose of Sappan wood ethanol extract

Negative control was a group that received exposure to formaldehyde inhalation but did not get Sappan wood extract. The caspase 3 levels of the negative control groups appeared well above those of the group that received Sappan wood extract. The caspase 3 levels of the groups of SWE 100, SWE 400, and SWE1000 were almost the same, but based on the pattern showed, the higher the dose of Sappan wood extract, the lower the caspase 3 levels.

DISCUSSION

Effects Of Sappan Wood Extract On Blood Serum Caspase 3 Levels Of Rats Exposed To Formaldehyde Gas.

Formaldehyde is one source of ROS and free radicals. When ROS and radicals enter the cell, they will cause cell injury through three main reactions, namely lipid membrane peroxidation, cross-reaction with proteins, and DNA damage. Peroxidation of lipid membranes occurs due to oxidative stress [26]. Oxidative stress can trigger the rate of apoptosis [27], [28]. The underlying mechanism is initiated by excess ROS and free radicals that attack mitochondria as their primary target [29]. This disruption causes Bcl (anti-apoptotic protein) to leave the mitochondrial membrane, consequently will encourage increased membrane permeability to release cytochrome C from the mitochondrial intermembrane and bind to a cytoplasmic protein called Apaf-1. The bond between cytochrome C and Apaf-1 forms the caspase recruitment domain (CARD). Several

CARDS will then combine to form an apoptosome complex, then bind to pro-caspase 9 and activate caspase 9. Caspase 9 will activate pro caspase 3 so that caspase 3 is formed [30]. Although the mechanism of apoptosis for changes in the structure of the nasal epithelium is not known with certainty, the presence of caspase 3 is characteristic of apoptosis [31].

Cell injury through DNA damage mechanisms caused by the reaction of free radicals with thymine in the nucleus and mitochondrial DNA results in the breaking of single strands. Living cells have mechanisms to repair damaged DNA, but if the damage is too severe, such as after oxidative stress, living cells can initiate suicide programs through apoptosis [32]. Caspase 3 is a major component of the apoptotic cascade that activates DNA ase and induces DNA fragmentation [33].

Formaldehyde in this study, inducing a significant increase in caspase-3 levels. This was seen in the negative control group that had the highest caspase levels. The formation of apoptosis in rats exposed to formaldehyde gas has also been proven in several studies. The results of the analysis showed that there was a significant difference in caspase 3 levels among all groups. The SWE 1000 group showed significantly lower levels of caspase 3 than normal controls, but this was not the case in the SWE 400 and 100 groups. This study proves that giving SWE of 1000 mg/kg body weight/day can inhibit apoptosis activity directly.

The presence of caspase 3 in the normal control group (who were not exposed to formaldehyde) of 0.134050 pg/ml is one of the normal physiological phenomena to maintain cell safety. This is done to get rid of abnormal cells [34],[35] and limiting the rate of cell proliferation when environmental conditions are not favorable for cell development. This is important as a way of selection to eliminate cells that have genetic defects [36], [37].

Descriptively, when compared with the negative control group, the average caspase 3 level was decreased with increasing doses of Sappan wood extract. The caspase 3 level of groups who received a Sappan wood extract dose of 1000 mg was the same as those who received vitamin C. The increasing dose of Sappan wood extract, the lower the value of caspase 3 which showed a decrease in apoptosis events.

Sappan wood extract supplementation can suppress apoptotic cells, because sappan wood contains high flavonoids. Brazilin and Brazilein, are active natural compounds from sappan wood that are classified as flavonoids. Brazilin and Brazilein have been shown to exhibit anti-

inflammatory, antioxidant properties and inhibit the growth of some cancer cells. Brazilin scavenges free radicals and inhibits lipid peroxidation, and exhibits antioxidation activity [15].

Flavonoids that act as antioxidants capture oxidants directly (act as scavengers) thereby reducing cell death (apoptosis) [38]. Flavonoids also increase normal keratinocyte resistance through delay and inhibition of intrinsic apoptotic signaling [39]. Flavonoids can inhibit caspase-1, caspase 3, and caspase 7 and inhibition by some flavonoids can be specific to certain caspases. Flavonoids may provide an effective starting point for the development of certain molecular caspase inhibitors as therapeutic agents [40]. Consistent research shows flavonoids can inhibit staurosporine-induced caspase-3 or −7 activity [41]. Pinocembrin which is a natural flavonoid that can inhibit apoptotic activity is shown by decreased transcriptional regulation of caspase 3 [42]) and studies in formaldehyde-induced rats show a decrease in apoptosis rates with flavonoid administration [39]. These results suggest that flavonoids may provide an effective starting point for the development of caspase inhibitors as disease-preventive agents.

CONCLUSION

Administration of *Caesalpinia sappan L.* Extract as an antioxidant before exposure of rats to formaldehyde inhalation can suppress apoptosis. The dose of *Caesalpinia sappan L.* Extract 1000 mg / kg body weight / day is the best and gives the same results as vitamin C. Flavonoids in Caesalpinia sappan L may provide an effective starting point for the development of caspase inhibitors as disease-preventive agents.

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