Synergistic Protective Effect of Commercial Nigella Sativa Oil and Honey Combination against Cisplatin-induced Nephrotoxicity in Rats

Efek Protektif Sinergis Kombinasi Minyak Jintan Hitam (*Nigella sativa*) Komersial dan Madu terhadap Nefrotoksisitas pada Tikus akibat Cisplatin

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Abstract

Cisplatin (CP) is a widely used chemotherapeutic agent that induces nephrotoxicity. Nigella sativa oil (NSO) and honey (H) have demonstrated a nephroprotective effect. The aim of this research is to investigate the effect and synergy of NSO and honey combination on kidney malondialdehyde (MDA) levels in CP-induced rats. Thirty male rats were divided into ten groups: Normal Control (NC), NSO1, NSO2, H1, H2, NSO1-H1, NSO1-H2, NSO2-H1, NSO2-H2, and CP-treated group which received ad libitum diet, 1 mL/kg body weight (kgbwt) NSO, 2 mL/ kgbwt NSO, 3.7 mL/kgbwt H, 7.4 mL/kgbwt H, 1 mL/kgbwt NSO and 3.7 mL/kgbwt H, 1 mL/kgbwt NSO and 7.4 mL/kgbwt H, 2 mL/kgbwt NSO and 3.7 mL/kgbwt H, 2 mL/kgbwt NSO and 7.4 mL/kgbwt H, ad libitum diet, respectively, for 21 days. On the 18th day, NC group received 1 ml/kgbwt NaCl 0.9% while the rest received 8 mg/kgbwt single dose CP intraperitoneally. Kidney MDA level was measured using the thiobarbituric acid reactive substance (TBARS) method. Kidney MDA level was significantly lower in all single and combination dose treatment groups compared to the CP-treated group ($p<0.001$). The combination index of all combination dose treatment groups was <1. NSO and honey combination provided a synergistic protective effect against CP-induced oxidative damage in rat kidney tissue.

Keywords: Nigella sativa oil, honey, MDA, cisplatin, nephrotoxicity

Abstrak

Cisplatin (CP) merupakan agen kemoterapi yang banyak digunakan dan menyebabkan nefrotoksisitas. Minyak jintan hitam (MJH) dan madu (M) diketahui memiliki efek nefroprotektif. Tujuan penelitian ini adalah untuk mengetahui efek dan sinergitas kombinasi MJH dan madu pada kadar malondialdehid (MDA) ginjal tikus yang diberi pajanan CP. Penelitian ini menggunakan 30 ekor tikus jantan yang dibagi menjadi 10 kelompok: Kontrol Normal (KN) (diet ad libitum), MJH1 (1 mL/kgBB MJH), MJH2 (2 mL/kgBB MJH), M1 (3,7 mL/kgBB M), M2 (7,4 mL/kgBB M), MJH1-M1 (1 mL/kgBB MJH dan 3,7 mL/kgBB M), MJH1-M2 (1 mL/kgBB MJH dan 7,4 mL/kgBB M), MJH2-M1 (2 mL/kgBB MJH dan 3,7 mL/kgBB M), MJH2-M2 (2 mL/kgBB MJH dan 7,4 mL/kgBB M), dan CP (diet ad libitum). MJH dan madu diberikan secara oral selama 21 hari. Pada hari ke-18, KN diberikan NaCl 0,9% 1 ml/kgBB sedangkan sisanya diberikan CP dosis tunggal 8 mg/kgBB secara intraperitoneal. Kadar MDA ginjal diukur menggunakan metode thiobarbituric acid reactive substances (TBARS). Didapatkan kadar MDA ginjal yang lebih rendah pada semua kelompok dosis tunggal dan kombinasi dibandingkan dengan kelompok CP (p=0,000). Semua kelompok dosis kombinasi memiliki indeks kombinasi <1. Kombinasi MJH dan madu memberikan efek protektif sinergis terhadap kerusakan oksidatif jaringan ginjal tikus akibat pajanan CP.

Kata kunci: *Minyak jintan hitam, madu, MDA, cisplatin, nefrotoksisitas*

Introduction

International Agency for Research on Cancer (IARC) reported that the global burden of cancer has risen to 18.2 million new cases and 9.6 million deaths by 2018 and is expected to increase in the future.[1] Chemotherapy still remains as one of the most common cancer treatments available nowadays. Cisplatin (CP), also known as cis-diamine dichloroplatinum (II), is a platinum-based inorganic chemotherapeutic drug that has been extensively used for various types of cancer such as lung, ovarian, testicular, breast, brain cancer, and also head and neck squamous cell carcinoma (HNSCC).[2]

CP exhibits anti-cancer activity through multiple cytotoxic mechanisms and is best known for causing DNA damage. It also causes cytoplasmic organelle dysfunction, particularly with the endoplasmic reticulum and mitochondria. Also, CP activates apoptotic pathways and inflicts cellular damage via oxidative stress and inflammation. Even though CP has a suppressive effect against various types of cancer, it has serious side effects that limit its use as a chemotherapeutic drug. Nephrotoxicity is reported to be its major side effect manifested as acute kidney injury which is seen in approximately 20–30% of the patients undergoing CP treatment.[3]

Nigella sativa (NS) is a widely used medicinal plant all over the world. Its seeds and oil have been used in the treatment of different diseases. It is an enriched source of nutritionally essential constituents and its oil (NSO) is rich in polyunsaturated fatty acids (PUFA), phytosterols, and several other phytochemicals including thymoquinone that exhibits strong antioxidant properties.[4] NSO is reported to be protective against CP-induced nephrotoxicity. It is protected from CP-induced free radical attack by activating the endogenous antioxidant defense mechanism.[5]

Honey (H) is a natural food product, which next to its nutritional importance, possesses valuable therapeutic properties due to the presence of bioactive ingredients. Flavonoids and phenolic acid constituents have been reported to be solely responsible for the antioxidant of honey by acting as free radical scavengers, through the formation of more stable and less toxic molecules.[6] Moreover, ascorbic acid, α-tocopherol, carotenoids, and other enzymes (catalase, glucose oxidase, and peroxidase) found in honey also contribute to its antioxidant capacity.[7] A study reported that honey has demonstrated a protective effect against CP-induced nephrotoxicity through suppression of inflammation which may be related to reduction of oxidative stress.[8]

Malondialdehyde (MDA) is a secondary product of lipid peroxidation produced from polyunsaturated fatty acids (PUFAs) by both chemical reactions and reactions catalyzed by enzymes. It is the most frequently measured biomarkers of oxidative stress, namely of lipid peroxidation.[9] Increased MDA levels in the body indicate an increased lipid peroxidation as a result of CP exposure.[5]

Several studies have reported that NSO[5] and honey[10] demonstrated protective effect and was able to reduce malondialdehyde (MDA) levels when used individually. It is also reported that their use in combination provided more effective protective effect.[11] Therefore, the present study aims to investigate the effect and synergy of NSO and honey combination on kidney MDA levels in CP-induced rats.

Methods

The randomized post-test only control group experimental design was conducted in a nonmicroscopic laboratory, faculty of medicine, Tanjungpura University in October 2018 - February 2019.

1. Drugs and chemicals

NSO was purchased from PT. Habbatussauda International with a trademark of "Habbat's Blackseed Oil" and registered BPOM number TR083697951. Honey by *Trigona incise* (obtained from Paloh, Sambas, Kalimantan Barat) was purchased from Rumah Madu Syaiful with a trademark of "*Madu Syaiful*" and registered SPP-IRT number 2096171010256-21. Cisplatin (CP) was purchased from PT. Dankos Farma (Kalbe Company). *Malondialdehyde bis-(dimethyl acetal)* (catalogue number 8207560250), Trichloroacetic acid (TCA) (catalogue number 1008070250), Thiobarbituric acid (TBA) (catalogue number 1081800025), and Glacial acetic acid 100% (catalogue number 1000632500) were all from Merck. Analytical reagents grade chemicals with high purity were used for this study.

2. Animal protocol

Thirty male Wistar rats weighing 180-220 g (2-3 months old) were allowed to acclimatize for one week before the experiment. All animal experiments were performed following the institutional guidelines and were approved by the Ethics Committee of Tanjungpura University (No: 2611/UN22.9/DL/2018). The animals were divided into 10 groups consisting of 3 rats per group. Normal Control (NC), NSO1, NSO2, H1, H2, NSO1-H1, NSO1-H2, NSO2-H1, NSO2-H2, and CP-treated (CP). Rats were fed an *ad libitum* diet, 1 mL/kg body weight (kgbwt) NSO, 2 mL/kgbwt NSO, 3.7 mL/kgbwt H, 7.4 mL/kgbwt H, 1 mL/kgbwt NSO and 3.7 mL/kgbwt H, 1 mL/kgbwt NSO and 7.4 mL/kgbwt H, 2 mL/kgbwt NSO and 3.7 mL/kgbwt H, 2 mL/kgbwt NSO and 7.4 mL/kgbwt H, *ad libitum* feeding by oral gavage was given once a day during the 21-day experimental period. On the 18th day of the experiment, NC group received 1 mL/kgbwt NaCl 0.9% while the rest of the treatment groups received 8 mg/kgbwt single dose CP intraperitoneally. The doses of NSO and honey were selected based on the previous studies which used 2 ml/kgbwt NSO[5] and 5 g/kgbwt honey[12], while the dose of CP was selected based on its recommendation dose for treating cervical cancer stage IB and IIA.[13]

Rats were sacrificed on day 22nd. The kidneys were immediately excised, washed in ice-cold normal saline, and then stored at -20^{o}C.

3. Preparation of homogenates

The kidneys were decapsulated and weighed. A hundred mg of kidney tissue was kept in cold phosphate buffer saline (0.1 M, pH 7.4) and then homogenized using *Tissue Lyser* II. It was then vortexed and centrifuged at 10.000 rpm for 10 minutes at 4°C. The supernatant was stored at -20°C prior to analysis.

4. MDA measurement

Determination of MDA level in kidney tissue was determined according to the method of Buege and Aust[14] in Esterbauer and Cheeseman's later modification.[15] This method is based on the reaction with thiobarbituric acid (TBA) in an acidic pH at 90–100ºC. In the TBA test reaction, MDA or MDA-like substances (produced during lipid peroxidation) and TBA react with production of a pink pigment with a 532 nm absorption maximum.

Three hundred µL of sample supernatant was added to 300 µL of 20% TCA. The mixture was vortexed and centrifuged at 3.000 rpm for 10 minutes at 4°C. Five hundred µL of supernatant was added to 300 µL of 1% TBA (prepared in 50% glacial acetic acid) and the mixture was incubated in a boiling water bath for 10 minutes. After cooling, the absorbance was read at by the UV-vis spectrophotometer (Genesys 10s) at 532 nm against blank using distilled water. The concentration of MDA in kidney tissue was expressed as nmol MDA/ml.

5. Statistical analysis

The recorded parameters were expressed as means and standard deviation (mean ± SD) for all groups and statistically analyzed using SPSS (version 23) software, for the one-way analysis of variance (one-way ANOVA) followed by Post Hoc Least Significant Difference (LSD) test. A probability level of $p<0.05$ was selected as an indicator of statistical significance.

6. Combination index (CI) analysis

CI value of NSO and honey combination was analyzed using Compusyn 2005 for windows which are interpreted as follows:[16]

a. CI < 0.1: very strong synergism
b. CI 0.1−0.3: strong synergism
c. CI 0.3−0.7: synergism
d. CI 0.7−0.85: moderate synergism
e. CI 0.85−0.90: slight synergism
f. CI 0.90−1.10: nearly additive
g. CI 1.10−1.20: slight antagonism
h. CI 1.20−1.45: moderate antagonism
i. CI 1.45−3.3: antagonism
j. CI 3.3−10: strong antagonism
k. CI >10: very strong antagonism

Results

1. Effect of CP, NSO, Honey, and Combined NSO-honey Administration on Kidney MDA Levels in CP-induced Rats

Results of this study (summarized in Figure 1) showed a single dose of CP administration as seen in CP-treated group, significantly increased lipid peroxidation in kidney tissue (measured as kidney MDA levels) compared to NC group ($p<0.05$). Either NSO or honey administered individually prior to CP administration as seen in NSO1, NSO2, H1, and H2-treated groups, significantly decreased the CP-induced lipid peroxidation level compared to CP-treated group ($p<0.05$). Furthermore, administration of both NSO and honey in combination prior to CP administration as seen in NSO1-H1, NSO1-H2, NSO2-H1, and NSO2-H2-treated groups, was more effective in reducing kidney lipid peroxidation level compared to the CP-treated group, with NSO2-H2 being the most effective combination dose which decreased the MDA levels to nearly the control ranges ($p=0.307$).

2. Combination index (CI) of NSO and honey Combination

CI value of all NSO and honey combination dose treatment groups exhibited synergistic effect (Table 1). NSO1-H1, NSO1-H2, and NSO2-H1-treated groups exhibited strong synergism while NSO2-H2-administered group exhibited very strong synergism.[16]

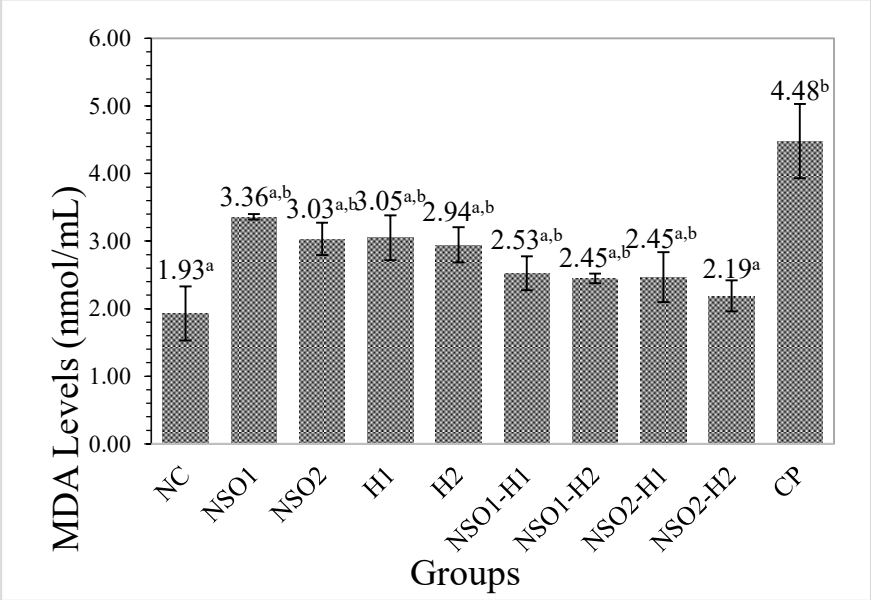

Figure 1. Effect of CP, NSO, honey, and Combined NSO-honey Administration on Kidney MDA Levels in CP-induced Rats.
Results are mean ± SD for three different samples (n =3).
[a]: Significantly different from CP-treated group.
[b]: Significantly different from NC group.

Table 1
Combination Index (CI) Value of NSO and honey Combination

Combination Groups	Treatment Doses (mL/kgbwt.)		CI Value	Descriptions
	NSO	H		
NSO1-H1	1	3.7	0.17	Strong synergism[16]
NSO1-H2	1	7.4	0.13	Strong synergism[16]
NSO2-H1	2	3.7	0.23	Strong synergism[16]
NSO2-H2	2	7.4	0.08	Very strong synergism[16]

Discussions

A significant elevation of kidney MDA levels in the present study indicated that CP administration increased lipid peroxidation, thus causing oxidative damage in kidney tissue in rats. This finding is an agreement with earlier studies.[5,17] Farooqui *et al*[5] reported that CP administration significantly enhanced lipid peroxidation as evidenced by increased MDA levels and decreased GSH and total protein thiols (-SH), suggesting CP-induced oxidative stress. A study conducted by Abdel-Wahab *et al*[17] confirmed that administration of CP increased kidney MDA levels and suppressed the activity of the major antioxidant enzymes in kidney (GPx, GSH reductase/GR, SOD, and CAT), making the kidney more vulnerable to damage by oxygen radicals. The decline in the enzymatic antioxidants, along with the increase in lipid peroxidation, indicates that the enzymes are being consumed in combating the increased free radical production in renal tissue. The change in

oxidant/antioxidant balance following CP administration points out to a state of oxidative stress in the kidney, which might be responsible for the observed perturbations in the biochemical markers.[17]

In this study, administration of NSO individually protected against CP-induced oxidative damage. This was evidenced by decreased kidney MDA levels. Farooqui *et al*[5] explained that NSO may have exerted its protective effect against CP-induced oxidative damage by scavenging free radicals, thus inhibiting generation of new free radical; increasing both the levels and activities of not only endogenous antioxidants but also antioxidant enzymes, thus strengthening kidney antioxidant defense system; and replacing PUFA components of membranes that had been previously attacked by reactive oxygen species (ROS), thus accelerating the process of reparation/regeneration of the damaged membranes. Also, Alsuhaibani[18] observed that all powder, oil, and extracts of NS prevented CP-induced nephrotoxicity via direct and indirect antioxidant activity from induction of endogenous antioxidant enzymes.

Alkadri[19] and Chikrista[20] in the previous studies conducted a phytochemical analysis of NSO and reported the presence of quinone compounds. Quinone derivative, Thymoquinone (TQ), is the most important active compound found in NSO which acts as an antioxidant. It also exhibits cytoprotective effect that protects kidney from oxidative damage by inhibiting lipid peroxidation *in vitro* (measured as MDA) and increases the level of enzymatic antioxidants (SOD, CAT, GPx) in kidney tissue.[21]

Thymohydroquinone and glutathionyldihydro-TQ are the reduced form of TQ containing two active hydroxyls (-OH) group in their structure, are thought to be responsible for antioxidant capacity.[22] Badary *et al*[23] reported that TQ mainly acted as a potent superoxide anion scavenger. TQ also showed the ability of scavenging hydroxyl radical, thus inhibited propagation of lipid peroxidation.

Administration of honey individually in the present study showed a protective effect against CP-induced oxidative damage. Hamad *et al*[8] demonstrated that oral administration of honey effectively prevented CP-induced nephrotoxicity by reducing inflammation and oxidative stress through inhibition of nuclear factor-kappa B (NF-κB) activation. The protective effect of honey against nephrotoxicity induced by oxidative stress-producing agents, also confirmed by Ali et al[10], as represented by the reduction of serum MDA levels and elevation of serum GSH levels with improvement of the kidney histological findings. This is presumably due to the free radical scavenging property in honey which interfered with oxidative stress process.[10]

Phytochemical analysis of honey conducted by Alkadri[24] and Chikrista[20] revealed the presence of flavonoids and phenolic acids (nonflavonoids) that belong to the main classes of phenolic compounds. Flavonoids found in honey are apigenin, genistein, pinocembrin, chrysin, quercetin, luteolin, galangin, and pinobanksin while phenolic acids in honey include 4-(Dimethylamino) benzoic acid, caffeic acid, p-coumaric acid, gallic acid, vallinic acid, syringic acid, and chlorogenic acid. These compounds are responsible for the antioxidant activity of honey by acting as free radical scavengers, through the formation of more stable and less toxic molecules.[6]

In general, phenolic compounds act as antioxidants by several mechanisms. Phenolic hydroxyl groups can break the cycle of new free radicals generation by donating hydrogen that reacts with reactive oxygen and reactive nitrogen species in termination reactions. Phenolics can inhibit some enzymes involved in radical

generation, such as various cytochrome P450 isoforms, lipoxygenases, cycloxygenases, and xanthine oxidase. They also can chelate metal ions involved in the production of free radicals.[25]

In addition, ascorbic acid, α-tocopherol, carotenoids, and other enzymes (catalase, glucose oxidase, and peroxidase) found in honey also attribute to its antioxidant capacity.[7]

Vitamin E (α-tocopherol), is an efficient lipid soluble antioxidant that functions as a 'chain breaker' during lipid peroxidation in cell membranes and various lipid particles including low-density lipoprotein (LDL). Vitamin C or ascorbic acid is a water-soluble free radical scavenger. Moreover, it regenerates vitamin E in cell membranes in combination with GSH or compounds capable of donating reducing equivalents. Carotenoids are among the most common lipid-soluble phytonutrients. Carotenoids are known to play an important role in the protection of cellular membranes and lipoproteins against the ROS due to their peroxyl radical scavenging activity.[26]

In the present study, administration of NSO and honey in combination protected against CP-induced oxidative damage more effectively compared to each of them when administered individually. Combination of 2 mL/kgbwt NSO and 7.4 mL/kgbwt honey in this study exhibited the most effective protective effect resulted in significant reduction of MDA level to nearly the control ranges. This result is in accordance with Abdallah *et al*[11] who reported that administration of NS and honey in combination provided more effective protective effect compared to either agent administered individually against paracetamol (PCM)-induced hepato-renal toxicity in rats, as evidenced by decreased the liver enzymes, improved kidney functions, increased tissue total antioxidant capacity (TAC) to nearly the control ranges, and, and decreased serum FasL. Furthermore, Mabrouk *et al*[27] reported that NS grains have given orally protected against *methyl nitrosourea* (MNU)-induced oxidative stress and carcinogenesis by 80% and combated this effect by lowering MDA and nitric oxide (NO) level whereas NS and honey administered together protected against MNU-induced oxidative stress and carcinogenesis by 100%.

CI analysis showed that NSO1-H1, NSO1-H2, and NSO2-H1-administered groups exhibited strong synergism while NSO2-H2-administered group exhibited very strong synergism. Mohamad *et al*[28] demonstrated that Trolox Equivalent of the lipophilic antioxidant capacity of the mixture of NS seeds and honey was higher than either NS seeds or honey individually which indicated that the mixture of NS seeds and honey demonstrated the highest antioxidant capacity. These findings suggested that lipid-soluble antioxidants in whole seeds of NS have a synergistic effect when combined with antioxidants in honey, thus causing a higher antioxidant capacity for the mixture as compared to either NS seeds or honey individually. In addition, Odobasic *et al*[29] also determined antioxidative capacity of NSO, honey, and the mixture of NSO and honey using Ferric Reducing Antioxidant Power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods and confirmed that the mixture of NSO and honey in relation 3:2 has a higher concentration of total phenols in relation to pure honey.

TQ[22] is a lipophilic molecule whereas phenolic compounds[30] can be either hydrophilic or lipophilic. The findings of present study suggested that the variation of bioactive constituents found in NSO and honey when combined might have a wider antioxidant network which resulted in synergism. Correspondingly, Yeum *et al*[31] reported that *in vitro* study indicated that physiologic concentrations of antioxidants located in the aqueous and lipid compartments synergistically interact to protect against oxidation of

reconstituted human serum. Physiologic doses of water- and fat-soluble antioxidant nutrients may be required to establish an effective antioxidant network *in vivo*. Moreover, Chen *et al*[32] clarified that drug combinations, with multiple targets belonging to interlinked processes, can combat the systematic pathological states through the cooperative mechanism and that synergistic drugs tend to have effects on targets with distinguishing network features.

Conclusions

The present study confirmed that commercial NSO and honey administered either individually or in combination protected against CP-induced oxidative damage. This study also indicated that 2 mL/kgbwt NSO and 7.4 mL/kgbwt honey in combination provided the most effective protective effect. The finding of this study suggested that combination of NSO and honey provided synergistic protective effect.

Recommendations

We recommend doing further researches on effect of NSO and honey combination in CP-induced exposed rats using antioxidant parameters such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx).

Acknowledgements

The authors gratefully acknowledge Sumo Lestari, ST, Nurhidayati, A. Md, and Amdalia Sri Swastiastuti for their technical help in conducting this research.

Funding

This research was partially funded by Community Development (Comdev) and Outreaching unit of Tanjungpura University.

Conflict of Interest Statement

None.

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