

IgM antibody and colony fungal load impacts of orally administered ethanol extract of *Plectranthus scutellarioides* on mice with systemic candidiasis

[Impacto de la administración oral del extracto etanólico de *Plectranthus scutellarioides* sobre anticuerpos IgM y la carga fúngica en ratones con candidiasis sistémica]

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Abstract

Context: *Candida albicans* is a pathogenic fungus that causes vulvovaginal candidiasis (VVC), which presents significant clinical problems in immunocompromised patients. There are no studies that correlate IgM antibody responses and fungal loads following the use of ethanol leaf extract of *Plectranthus scutellarioides* (L.) R.Br. (ELEP) on VVC.

Aims: To examine colony fungi loads, IgM antibody responses, and their correlation in Balb/c mice infected with *C. albicans* treated with orally administered ELEP.

Methods: Twenty-five female Balb/c mice were randomly divided into five groups (five mice in each group): normal group (oral ELEP + not infected), VVC control (VCC) group (infected + oral 0.2 mL saline), ketoconazole-treated in VCC group (infected + oral antifungal agents), and two treatment groups (infected + oral ELEP with 500 and 750 mg/kg b.w.). Treatments were administered for 14 days after infection with *C. albicans*. Colony fungal loads and IgM antibodies were measured by counting colony forming units and flow cytometry using anti-*C. albicans* antibody kits, respectively.

Results: Ingestion of the ELEP significantly affected fungal load clearance and decreased IgM antibody levels compared to that in the VCC group ($p < 0.001$). Furthermore, ingestion of the ELEP was associated with significantly lower concentrations of IgM antibodies and colony forming units ($p < 0.001$).

Conclusions: These data strongly suggest that IgM antibody response is involved in of the mechanism by which ELEP decreases *C. albicans* infection. Furthermore, these results provide important information for the potential application of ELEP in the treatment of Balb/C mice with systemic candidiasis.

Keywords: anti-candida; immune response; miana leaves; vulvovaginal candidiasis.

Resumen

Contexto: *Candida albicans* es un hongo patógeno que causa candidiasis vulvovaginal (VVC), que presenta problemas clínicos significativos en pacientes inmunocomprometidos. No existen estudios que correlacionen las respuestas de anticuerpos IgM y cargas fúngicas después del uso del extracto etanólico de hoja de *Plectranthus scutellarioides* (L.) R.Br. (ELEP) en VVC.

Objetivos: Examinar la carga fúngica de colonias, la respuesta de anticuerpos IgM y su correlación en ratones Balb/c infectados con *C. albicans* tratados con ELEP administrado por vía oral.

Métodos: Veinticinco ratones Balb/c hembras se dividieron en cinco grupos (cinco ratones/grupo): grupo normal (ELEP oral no infectado), grupo control VVC (infectado + 0,2 mL salino oral), tratado con ketoconazol en el grupo VCC (VVC + agentes antifúngicos orales) y dos grupos de tratamiento (VVC + ELEP oral con 500 y 750 mg/kg p.c.). Los tratamientos se administraron durante 14 días después de la infección con *C. albicans*. Las cargas fúngicas y los anticuerpos IgM se midieron contando unidades formadoras de colonias y citometría de flujo usando kits de anticuerpos anti-*C. albicans*, respectivamente.

Resultados: La ingestión de ELEP afectó el aclaramiento de la carga fúngica y la disminución de los niveles de anticuerpos IgM en comparación con el grupo VCC ($p < 0,001$). Además, la ingestión de ELEP se asoció con concentraciones más bajas de anticuerpos IgM y unidades formadoras de colonias ($p < 0,001$).

Conclusiones: Estos datos sugieren que la respuesta de anticuerpos IgM está implicada en el mecanismo por el cual ELEP disminuye la infección por *C. albicans*. Además, estos resultados proporcionan información importante para la aplicación potencial de ELEP en el tratamiento de ratones Balb/c con candidiasis sistémica.

Palabras Clave: anti-candida; candidiasis vulvovaginal; hojas de miana; respuesta inmune.

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INTRODUCTION

Candida albicans, a polymorphic fungus, is a member of the normal human microbiome (Mayer et al., 2013). They are, however, the most common cause of fungal disease in humans (Karkowska-Kuleta et al., 2009), often causing supercritical infection, because *C. albicans* can colonize the mucosal surfaces of healthy people and thus occurs commensally in the gastrointestinal tract, oral cavity, and vagina (Mavor et al., 2005). Moreover, *C. albicans* can cause life-threatening systemic infections, making it the most prevalent fungal pathogen of humans (Correia et al., 2016). Life-threatening *C. albicans* infections are a significant clinical problem, especially in immunocompromised individuals (Shahin et al., 2016). There are two mechanisms by which *C. albicans* can enter the bloodstream in humans: direct penetration from the epithelium after tissue damage (Mavor et al., 2005) and dissemination from biofilms formed on medical devices introduced into the patient (such as catheters, dental implants, and intravenous lines) (Chandra et al., 2001). Vulvovaginal candidiasis (VVC) is one of the common forms of mucosal candidiasis (Correia et al., 2016), and it estimated that approximately 70-75% of women suffer at least one episode of VVC in their lifetime (Sobel, 2007). Approximately 40-50% of these women also experience a recurrence (Sobel, 2007). A single strain causes frequent recurrences through subsequent infections (Sampaio et al., 2003). Risk factors for VVC include human immunodeficiency virus (HIV) infection (Duerr et al., 2003), diabetes, antibacterial vaginal, and immunogenetic alterations (Jaeger et al., 2013).

Azole antibiotics are typically the treatment of choice for VVC but are limited by the development of azoles antibiotic resistance (Odds, 1993; Sanglard and Odds, 2002). Therefore, many scientific researchers seek plant-derived extracts and related metabolites due to their efficacy effects, especially for anti-*Candida* (Costa et al., 2016; Salari et al., 2016; Sadowska et al., 2017). Plant-derived drugs and related metabolites have been reported to be safe and without side-effects (Obici et al., 2008; Toyang et al., 2012; Mafioletti et al., 2013). In Indonesian traditional medicine, especially in Toraja communities, *Plectranthus scutellarioides*

(L.) R.Br. (referred to as 'miana' in Indonesia) leaves are used to treat vaginal discharge and may therefore have anti-candidiasis properties. *P. scutellarioides* has antibacterial and antifungal properties (Grosvenor et al., 1995). To date, there are no studies on the use of *P. scutellarioides* as an anti-candidiasis treatment that correlated immune responses with decreased of colony fungal counts. This study performed to estimate the colony fungi loads, IgM antibody responses, and their correlation in Balb/c mice infected with *C. albicans* and treated with orally administered ethanol leaf extract of *Plectranthus scutellarioides* (L.) R.Br. (ELEP).

MATERIAL AND METHODS

Yeast strain and reagents

C. albicans standard strain (ATCC 10231) was used throughout this study from Abcam (Abcam Biochemicals, USA) and prepared according to factory instruction. The final preparation was suspended in sterile Dulbecco's phosphate-buffered saline (DPBS) (Sigma Chemical Co., St. Louis, Mo, USA) before use. All solvents and reagents were used analytical grade.

Plant material and extraction

The leaf of *P. scutellarioides* was collected from Indonesian Spices and Medicinal Crops Research Institute, West Java, Indonesia in January 2016 (6°34'37.95" S; 106°47'20.37" E; 224.64 m a.s.l.). The leaf of *P. scutellarioides* was identified by Dr. Joem Setijo Rahajoe at the Herbarium Bogoriense of the Indonesia Institute of Sciences (LIPI), Bogor, West Java, Indonesia. A voucher specimen was deposited at the Herbarium Bogoriense (number 2395). The extraction method was conducted based on solvent maceration (Nurcholis et al., 2016) with slight modification. Briefly, the air-dried powdered leaves of *P. scutellarioides* (one kg) were extracted with 96% ethanol (1:5 w/v) at room temperature for 24 h. The mixture was filtered through Whatman 4 paper and evaporated to dryness under vacuum with an evaporator (BUCHI, R-250, Switzerland). These extracts were then used for analysis.

Animals

Twenty-five 8-to-12 week female Balb/c mice were obtained from Laboratory Molecular Biology and Immunology, Faculty of Medicine, Hasanuddin University, Makassar City, South Sulawesi, Indonesia. The mice were housed in standard environmental conditions and fed with rodent standard diets and water *ad libitum* at the animal research unit of Hasanuddin University. All procedures used in these studies were approved by the Animal Care and Use Committee at Faculty Medicine, Hasanudin University (UH16010010 on March 7, 2016). Mice were anesthetized by inhalation with ether and dislocated by stretching of the spinal cord. A minimum number of animals were used to obtain reliable results, and all animals were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996.

Intravaginal infection with *Candida albicans*, colony fungal load and IgM antibody responses

Mice were infected intravaginally with *C. albicans* suspended in DPBS (1×10^2 yeast cell/mouse), except for the normal group. Twenty-five female Balb/c mice were divided into five groups: the VVC control (VCC, $n = 5$) received 0.2 mL saline orally; the ketoconazole-treated VCC group (VCK) received ketoconazole (200 mg/kg b.w. given orally); the ELEP500 and ELEP750 groups were orally given doses of 500 mg/kg b.w. and 750 mg/kg b.w., respectively. The doses were selected based on previously published studies and traditional use (Pakadang et al., 2015). The treatment was done in 1 to 14 days after infection the mice given orally. After anesthesia by inhalation with ether, the spinal cords of the mice were dislocated by stretching. Colony fungi loads and IgM antibody responses were monitored for 14 days. The colony fungal load was examined by taking a vaginal swab, culturing, and counting the number of resulting *C. albicans* colonies (Conti et al., 2014); results are shown as colony forming units (CFU) in the vaginal fluid (mL). Serum was collected from the submaxillary vein in mice and used for IgM analysis. The concentrations of IgM antibody were measured using ELISA kits for anti-*Candida albicans* (ab53891, Abcam, USA) according to

manufacturer's protocol. The serum samples were measured using a microplate reader (Biotek, USA) at 450 nm, and IgM antibody expressed in pg/mL according to a standard curve.

Chemical characterization

The presence of different secondary metabolites like alkaloids, flavonoids, saponins, phenols, tannins, glycosides, triterpenes, and steroids in the ethanolic extract of *P. scutellarioides* were analyzed by using standard phytochemical procedures described by Waras et al. (2015). The color intensity or the precipitate formation was used as analytical responses to these tests. Briefly, Meyer's, Wagner's and Dragendroff reagents were used for alkaloid. Mg^{2+} and HCl in ethanol were used for flavonoid. Foam test was used for saponins. $FeCl_3$ 1% was used for phenol and tannins test. Keller-Killani test was used for glycosides. Lieberman-Burchard reagents was used for triterpenes and steroids test.

Statistical analysis

Data is shown as mean \pm SEM. Comparison between groups was analyzed by one-way ANOVA with the Tukey's multiple comparisons test; a p -value < 0.05 was considered statistically significant. Pearson correlation with simple model regression was used to assess the association between level of colony fungal load and IgM antibody responses.

RESULTS

The first secondary metabolites screening confirmed the presence of alkaloid, glycoside, saponins, triterpenes, flavonoids, and phenols in the crude ethanolic extract of *P. scutellarioides* (Table 1). Secondary metabolites of steroids and tannins were absent from the crude ethanolic extract of *P. scutellarioides* (Table 1).

An anti-candidiasis study was done on mice infected with *C. albicans*; the mice were orally administered a crude ethanolic extract of *P. scutellarioides*. IgM antibody levels and colony fungal loads were monitored for 14 days; the results are shown in Figure 1. The highest IgM antibody response was 1.191 pg/mL from the VVC control (VCC) group, followed by *P. scutellarioides* extract of 500 mg/kg b.w (CSE500, 1.001 pg/mL), ketoconazole-treated VVC

group (VCK, 0.519 pg/mL), *P. scutellarioides* extract of 750 mg/kg b.w (CSE750, 0.518 pg/mL), and normal (0.217 pg/mL) groups (Fig. 1A). *P. scutellarioides* extract at the doses tested (500 mg/kg and 750 mg/kg b.w) decreased IgM levels compared with VCC. In both the ELEP750 and VCK groups, the decrease in IgM levels was significant ($p < 0.001$) compared to IgM levels in the VCC group at 14 days. Furthermore, IgM levels in the ELEP750 and VCK groups were not significantly different from the normal group 14 days. The results obtained from the present study of the effect of the ethanolic extract on decreasing colony fungal loads are presented in Fig. 1B. The mice infected with *C. albicans* (VCC group) had significantly more colony fungal units per mL vaginal fluid (46.52 CFU/mL) ($p < 0.001$) compared to the VCK and ELEP750 groups, with values of 18.00 CFU/mL and 18.92 CFU/mL, respectively.

Table 1. Secondary metabolites screening of the ethanolic extract of *P. scutellarioides*.

Name of secondary metabolites	Observation
Alkaloid	+
Glycoside	+
Saponins	+
Triterpenes	+
Steroids	-
Flavonoids	+
Phenols	+
Tannins	-

(+) = presence of metabolites; (-) = absence of metabolites

A positive correlation was seen between the IgM antibody response and colony fungal loads after *C. albicans* infection and treatment with a crude ethanolic extract of *P. scutellarioides* (Fig. 2). As shown in Fig. 2 A-D, the Pearson correlation coefficient and p values between IgM antibody response and colony fungal load were 0.9745 and < 0.0001 in the VCC group, 0.9307 and < 0.0001 in the VVK group, 0.9598 and < 0.0001 in the ELEP500 group, and 0.9168 and < 0.0001 in the ELEP750 group. These findings demonstrated significant decrease in colony forming units and IgM antibody levels in the

ELEP750, ELEP500 and VVK groups when compared with the VCC group.

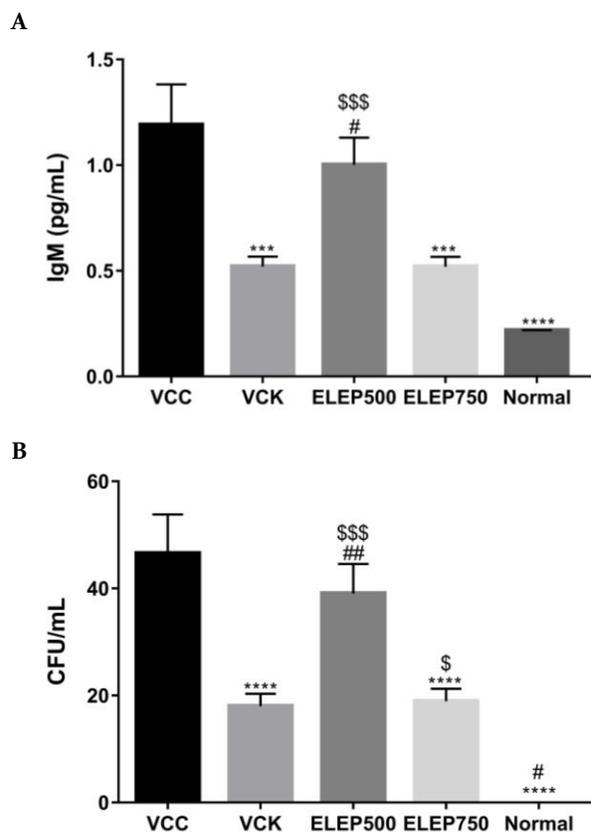


Figure 1. Potency of ethanol leaf extract of *P. scutellarioides* (ELEP) on IgM antibody response (A) and decreased colony fungal loads (CFU) level (B).

Mice were infected with *C. albicans* (1×10^2 CFU/mouse) intravaginally, except in the normal group (received 500 mg/kg ELEP b.w. and saline). Vulvovaginal candidiasis control (VCC) received saline; ketoconazole-treated in the vulvovaginal candidiasis group (VCK) received ketoconazole (200 mg/kg b.w.); the ELEP groups were orally given doses of 500 mg/kg b.w. (ELEP500) and 750 mg/kg b.w. (ELEP750). Values presented are the mean \pm SEM, $n = 5$. This experiment terminated at day 14. *** and ****, differed significantly from VCC at $p < 0.001$ and $P < 0.0001$, respectively; # and ##, differed from VCK at $p < 0.05$ and $p < 0.01$, respectively; \$ and \$\$\$, differed from normal at $p < 0.05$ and $p < 0.001$, respectively.

DISCUSSION

C. albicans is a major fungal pathogen of humans, and mortality among infected patients is high (Karkowska-Kuleta et al., 2009). VVC is an acute inflammatory disease caused by *C. albicans* infection that causes clinical signs and symptoms such as intense pruritus, vaginal discharge, an erythematous vulva and dyspareunia (Cassone, 2015). VVC can be

caused by multifactorial issues, such as genetic predisposition, pregnancy, contraceptives, diabetes mellitus, antibiotics; behavioral factors such as female hygiene habits; and other factors such as iron deficiency (Sobel, 2007). The azole antibiotics are the treatment of choice for VVC (Sobel, 2007; Chew and Than, 2016), but its use has various problems, including resistance, toxicity and side effects (Bodey, 1992; Cleary et al., 2013). Currently, many researchers are focusing on bioactive medicinal plants to find antifungal alternative for the treatment of candidiasis. Zhang et al. (2013) showed 15 extracts from 58 plant extracts possess anti-fungal activity for *C. albicans*. Also, five Indian medicinal plants viz. *Clerodendron colebrookianum* Walp. (leaf), *Gnetum gnemon* L.

(leaf), *Sarcochlamys pulcherrima* (Roxb.) Gaud. (leaf), *Garcinia lancifolia* (Don) Roxb (leaf) and *Euryale ferox* Salisb. (seed) showed anticandidal activity (Mazumder et al., 2012). This finding is important for the development of an effective VVC therapy.

In Indonesia, especially Minahasa communities, *P. scutellarioides* is traditionally used to treat vaginal discharge. This study was performed to evaluate the anticandidal activity of a crude ethanolic extract of *P. scutellarioides* in mice infected with *C. albicans*. Results showed that *C. albicans* yeast cells treated with *P. scutellarioides* extract (750 mg/kg b.w.) had 18.92 CFU/mL (Fig. 1), whereas untreated (VCC) yeast cells had 46.52 CFU/mL, resulting in a CFU reduction up to approximately 59%.

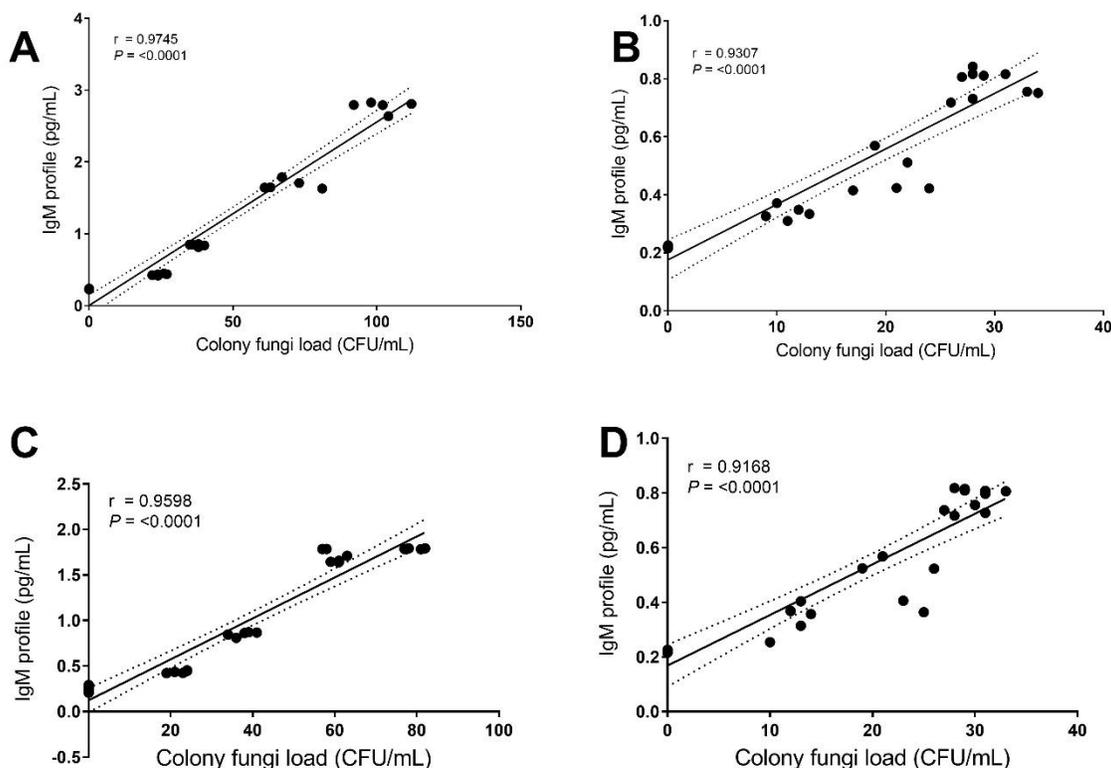


Figure 2. Scatter plot depicting the correlation between IgM antibody profile and colony fungal loads at vulvovaginal candidiasis control (A); ketoconazole-treated in vulvovaginal candidiasis group (B); and treatment groups of *P. scutellarioides* extract doses of 500 mg/kg b.w. (C) and 750 mg/kg b.w. (D).

The x-axis represents the *C. albicans* fungal load, while Y-axis represents the level of IgM antibody in pg/mL. Pearson's correlation coefficient (r) and p value are given in the separate graph. Each point corresponds to one group. The solid line exhibits the best line fitted to the data based on the simple regression model.

In addition, when treated with 500 mg/kg b.w (ELEP500) *P. scutellarioides* extract and ketoconazole (VVK), there was more than 16% and 61% CFU reduction, respectively. The data presented here indicate that ingestion of a ELEP was associated with significantly lower IgM antibody response at 14 days after infection of *C. albicans* (Fig. 1) compared with the VCC group. Consistent with their lower IgM antibody levels in serum, ELEP-treated mice also had lower fungal loads of yeast (CFU) at 14 days after infection. These findings are consistent with the traditional use of *P. scutellarioides* leaf. These results indicate that innate immunity is important as the first line to protect of the host against systemic candidiasis. It confirms results by Soltani et al. (2012) that macrophages have an important role in recognizing and eliminating invasive candidiasis. Previous studies have shown that various pharmacology activities for *P. scutellarioides* are associated with anticandidal activity. Pakadang et al. (2015) showed influence on the immune defense by oral administration of *P. scutellarioides* ethanolic extract in rats with resistance against tuberculosis infection. In addition, some researchers have also shown that potency of *P. scutellarioides* leaves as antimicrobial agents, such as the ethyl acetate fraction against *Staphylococcus aureus*, *C. albicans* and *Salmonella thyposa* (Effendi, 2010) and balm extract against scar infected *S. aureus* on the rabbit (Marpaung, 2014).

This study shows that the anticandidal activity of *P. scutellarioides* extracts (750 mg/kg b.w.) was the same potency with ketoconazole (200 mg/kg b.w.) for an enhanced killing of the *C. albicans* cells. Thus, ELEP and ketoconazole may exercise anticandidal activity via similar mechanisms of action through the inhibition of the fungal cytochrome P450, which is essential for the biosynthesis of ergosterol as a component of fungal plasma membrane (White et al., 1998). The ELEP contained flavonoid metabolite (Table 1); it may be another mechanism of anticandidal activity in ELEP. Some anticandidal properties of flavonoids as have been reported, such as inhibition of colony growth (Yoon et al., 2006; Herrera et al., 2010), inhibition of efflux pump and induction of apoptosis (Serpa et al., 2012; Zuzarte et al., 2012), cell wall damage (Sitheeque et al., 2009), and cytoplasmic membrane disruption (Zuzarte et al., 2012).

This result indicates the need to further identify bioactive compounds responsible for the anticandidal activity, especially in the treatment of VVC.

CONCLUSIONS

Positive correlations between fungal loads clearance and decreased IgM antibody levels have been found in mice infected with *C. albicans* and orally treated with ELEP. Thus, it suggested that the IgM immune response is involved in the mechanism by which ELEP decreases *C. albicans* infection. In an ethanolic extract of *P. scutellarioides*, there are potential sources of bioactive compounds; further study should be performed to identify the bioactive compounds responsible for the anticandidal activity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Contribution	Karo M	Hatta M	Patellongi I	Natzir T	Tambaip T
Concepts or ideas	X	X	X	X	X
Design	X	X	X	X	X
Definition of intellectual content	X	X	X	X	X
Literature search	X	X			
Experimental studies	X	X			
Data acquisition	X				
Data analysis	X				
Statistical analysis	X				
Manuscript preparation	X	X			
Manuscript editing	X	X			
Manuscript review	X	X	X	X	X

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