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Quality assessment of a decoction of *Sesamum indicum* L. and *Nigella sativa* L.: Polycystic ovary syndrome

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Abstract: Decoction prepared from *N. sativa*, and *S. indicum* can be used to treat oligo/anovulation, a primary sign of PCOS. Up to date no scientific experiments were carried out to access the quality of this decoction. Therefore, an attempt was made to evaluate the quality of the decoction made from *N. sativa* seeds and *S. indicum* seeds in terms of (a) phytochemical analysis (b) antioxidant activity (c) microbial limits (d) heavy metal limits and (e) development of TLC fingerprints using standard protocols. Results revealed that except flavonoids, other phytochemical constituents such as phenols, tannins, alkaloids, saponins, steroids, terpenoids, monoterpenes, sesquiterpenes and cardiac glycosides were present in the decoction. Total phenolic content of the decoction was 3.38 ± 0.07 mg gallic acid equivalents/g of extract and exhibited dose dependent (R^2 = 0.979) scavenging activity towards 1-diphenyl-2-picryl hydrazyl (DPPH) radicals. IC_{50} value of the decoction was 850.5±30.2 µg/mL for the DPPH assay. *Escherichia coli* and Coliforms were not detected whereas yeasts & moulds and *Staphylococcus aureus* were present (less than 10 colony forming units (CFU) per 1 ml of the decoction). Moreover, heavy metals such as Hg, As, Cd and Pb were not detected in the decoction. Further, TLC fingerprint profile was developed for the decoction and visualized the spots under UV light and calculated the R_f values of each spot. In conclusion, reported phytochemicals and antioxidant properties may be responsible for the remedy of Polycystic Ovary Syndrome while absence of harmful microbes and heavy metals are the indication of the safety of the decoction.

Keywords: Antioxidant, *Nigella sativa*, Polycystic Ovary Syndrome, *Sesamum indicum*

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INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is one of the most common endocrine disorders in women of reproductive age (Carmina & Lobo, 1999). PCOS prevalence varies through ethnicity and, estimates of the prevalence range from 2.2 % to 26 %. PCOS affects women of South Asian descent earlier age, with more severe symptoms and a higher prevalence. (Royal College of Obstetricians and Gynecologist Patient Information Committee, 2014). Oligo/anovulation, finding of multiple small cysts in ovaries, and features of excess androgen production such as hirsutism (excess facial or body hair), male or female pattern balding (hair loss), acne and acanthosis nigricans (skin discolorations) have been identified as the main signs and symptoms of Polycystic Ovary Syndrome (Boyle & Teede, 2012). *Sesamum indicum* L. and *Nigella sativa* L. have commonly used herbs in Ayurveda and traditional medicine for the treatment of common gynecological problems. Both herbs are used as single ingredient drugs (Anonymous, 1994) and as a formulation in Ayurveda medicinal system (Krishnedas, 1841 and Sastri, 1988) and are widely available at the local market as dried seeds. Both *S. indicum* and *N. sativa* seeds are used as drugs and spices and known locally as "Tala" and "Kaluduru," respectively. Both seeds are used to flavor Sri Lankan and Indian curries.

According to Yavari and co-workers (2016), *S. indicum* could be a good alternative to hormone therapy for oligomenorrhea. The sesame seeds therapy boosted FSH activity, according to Rahman and co-workers (Rahman et al., 2009). Further, *N. sativa* consist of substantial amount of the sex hormones such as estrogen, progesterone, prolactin, testosterone, FSH, and LH (Alta'ee et al., 2006). As per Modaresi and Poor-Naji (2012), a hydro-alcoholic extract of *N. sativa* can increase the number of follicles and corpus luteum in female mice, leading to increased fertility. Supplementing mice with *N. sativa* enhances oocyte quality and pre-implantation embryo development, resulting in greater reproductive performance (Mohammed and Farghaly, 2018; Mohammed, 2019). According to another animal research *N. sativa* is an alternative medication that may help women with PCOS with their menstrual irregularities (Naeimi et al., 2020) and have a prophylactic effect on the reproductive system of female mice (Kamarzaman et al., 2014).

Standardization of herbal drugs means quantifies the purity and quality of the drug. Today, the Ayurveda treatments are very popular in the world as most people believed that Ayurveda or herbal drugs have minimal side effects. Therefore, most are currently focused on methods of finding out the medicinal values of herbs and their use in the medicinal field. At the same time emphasis ensuring the quality of those drugs. The quality of herbal products is checked through stability testing studies which depend on various factors, such as chemical compounds, temperature, humidity, light, oxygen, moisture, other ingredients, microbial contamination, heavy metal contamination, etc. Many research have been carried out in Sri Lanka to set up the quality parameters for medicinal plants (Arawwawala and Arambewela, 2010; Arawwawala et al., 2011) and medicines (Hewageegana et al., 2013; Karunaratne et al., 2015).

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Standardization of powder forms, seed extracts of *N. sativa*, and *S. indicum* has been attempted, but no data on standardization of a decoction made with a combination of *N. sativa* and *S. indicum* is currently available. Therefore, an attempt was made to evaluate the quality of the decoction made from *N. sativa* seeds and *S. indicum* seeds in terms of (a) phytochemical analysis (b) development of TLC fingerprints (c) antioxidant activity (d) microbial counts and (e) heavy metal counts using standard protocols.

MATERIALS AND METHODS

Preparation of research drug: Seeds of *S. indicum* and *N. sativa* were purchased from an Ayurvedic drug supplier in Colombo district, Sri Lanka. The plant materials were identified, washed, dried, and decoction prepared (Figure 1) according to Ayurveda Pharmacopeia (Anonymous, 1994).

Figure 1. (a) Seeds of *Sesamum indicum* L. and *Nigella sativa* L. (b) Decoction made out of both seeds

Standardization of herbal decoction: Present drug was standardized in terms of phytochemical analysis, heavy metal limits, microbial limits, development of Thin Layer Chromatography (TLC) fingerprint and antioxidant screening as follows: *Phytochemical screening*: Phytochemical screening was carried out according to the established methods with some modifications (Yadav and Agarwala, 2011; Goveas and Abraham, 2014; Dahanayake et al., 2019).

Test for phenols: Folin reagent Test: a few drops of Folin reagent were added to 2 ml of decoction.

Ferric chloride Test: A few drops of Ferric chloride were added to 2 ml of decoction.

Test for Tannins:Lead acetate (1 ml from 10% solution) was added to 2 ml of decoction.

Test for Alkaloids: Picric acid test: Picric acid (1 ml) was added to 2 ml of decoction.

Tannic acid test: Tannic acid (1 ml) was added to 2 ml of decoction.

Test for saponins: Water (5 ml) was added to the decoction (1 ml) and shaken vigorously.

Test for steroids: Acetic anhydride (1 ml) was added to the decoction (2 ml) and mixed well. Then conc. H_2SO_4 (1 ml) was added to the mixture.

Test for terpenoids: Salkowski Test: Decoction (5 ml) was mixed with chloroform (2 ml). Then conc. H_2SO_4 (3 ml) was added.

Test for monoterpenes: Decoction (2 ml) was mixed with 10% vanillin in ethanol (2 ml). Then conc. H_2SO_4 (1 ml) was added.

Test for sesquiterpenes: Decoction (1 ml) was mixed with conc. H_2SO_4 (0.5 ml).

Test for cardiac glycosides: Decoction (5 ml) was mixed with glacial acetic acid (2 ml) and added 1 drop of $FeCl_3$. Then this was under layered with conc. H_2SO_4 (1 ml). A brown ring of the interface indicates cardiac glycosides.

Total polyphenolic content (TPC): Total polyphenol content of extract was determined by Folin-Ciocalteu spectrophotometric method (Singleton et al., 1999) using gallic acid as standard phenolic compound using 96-well microplates. Twenty microliters of four extracts, each dissolved in distilled water, were added to 110 µl of ten times diluted freshly prepared Folin-ciocalteu reagent and incubated with 70 µl of 10% sodium carbonate solution at room temperature (25 ± 2° C) for 30 minutes and the absorbance was recorded at 765 nm. Five different concentrations of gallic acid were used to construct the standard curve. Total Polyphenol content was expressed as mg gallic acid equivalents (GAE)/g of extract.

Total flavonoid content (TFC):Total polyphenol content of extract was determined by aluminium chloride method (Siddhuraju & Becker, 2003). One hundred microliters of 2% aluminium chloride in methanol solution was incubated with 100 µl of four samples dissolved in methanol at room temperature (25 ± 2° C) for 10 minutes and absorbance was recorded at 415 nm. Six different concentrations of Quercetin were used to construct the standard curve. Total Flavonoid Content was expressed as mg quercetin equivalents (QE)/g of extract.

Antioxidant activity via 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay:1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay was performed according to the method described by Blois (Blois, 1958). Herbal extract was tested at the assay

concentration range of 400–1200 µg/mL. DPPH solution (40 µg/mL, 200 µL) was incubated with plant extracts dissolved in methanol at room temperature (25 ± 2° C) in dark for 20 min. The absorbance was recorded against a blank at the wavelength of 517 nm. The capacity to scavenge the DPPH radical by 50% (IC_{50}) was calculated from the dose effect curves.

Microbiological Limits: Microbial counts in terms of aerobic plate count (Sri Lanka standards, 2013a), coliform count (Sri Lanka standards, 1991), yeasts and moulds count (Sri Lanka standards, 2013b), *Escherichia coli* (Sri Lanka standards, 2013c), Salmonella (Sri Lanka standards, 2013d), and *Staphylococcus aureus* (Sri Lanka standards, 1992) were carried out as per standard procedures detailed in Sri Lanka Standards.

Heavy metals: Quantitative determination of As (Official Methods of Analysis of AOAC International, 2012a), Hg (Official Methods of Analysis of AOAC International, 2012b), Cd (Official Methods of Analysis of AOAC International, 2012c) and Pb (Official Methods of Analysis of AOAC International, 2012a) was carried out according to Association of Official Agricultural Chemists (AOAC) methods using an Inductively Coupled Plasma Mass Spectrometry.

Extraction procedure: The herbal drug (20 g) was added to a round bottom flask which containing water (100 ml) and refluxed for 4 h. Then filtrate was added to a separatory flask containing 50 ml of dichloromethane and mixed well. Dichloromethane layer was separated. This was repeated thrice, and dichloromethane extracts were pooled and concentrated. Same procedure was followed to *S. indicum* seeds and *N. sativa* seeds.

Thin Layer Chromatography (TLC): TLC plates (20 cm x 20 cm) were cut into small plates (height:9.5 cm x width: 5 cm). A pencil line was drawn (base line) near 0.5 cm from the top to bottom of the plate. Care was taken not to press so hard with the pencil disturb the absorbent. Under the line, the names of the samples were marked lightly as A (for *N. sativa*), B (for herbal decoction) and C (for *S. indicum*). Microcapillary tube was dipped into each extract (*N. sativa* or herbal decoction or *S. indicum*) separately and taken 10 µl and then gently touched the end of the microcapillary onto the location (either A or B or C) of the TLC plate. Then the spotted TLC plate was dipped into the developing tank containing ethyl acetate, dichloromethane, and cyclohexane in a ratio of 0.5:5.5:1 (v/v), covered the tank with the watch glass and it was left undisturbed on a bench top. Plate was allowed to develop until the solvent was about half a centimeter below the top of the plate. The plate was removed from the solvent tank and allowed to dry. Then the place we observed under UV 254 nm and 366 nm. The spots were marked, and the place was sprayed with vanillin sulphuric acid, heated on a hot plate at 100° C for 5 minutes. Subsequently Spots were marked and Rf values were calculated.

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RESULTS AND DISCUSSION

Except flavonoids, other phytochemical constituents such as phenols, tannins, alkaloids, saponins, steroids, terpenoids, monoterpenes, sesquiterpenes and cardiac glycosides were present in the decoction (Table 1).

Table 1. Phyto-chemical constituents of the decoction prepared with seeds of *Sesamum indicum* L. and *Nigella sativa* L.

Phyto-chemical constituents	Test	Results
Phenols	Folin Reagent Test Ferric Chloride Test	Presence (Blue color)
Tannins	Lead Acetate test	Presence (Yellow precipitate)
Alkaloids	Picric acid test Tannic acid test	Presence (Yellow crystalline precipitate)
Saponins	Frothing test	Presence (Stable froth)
Steroids	Test for steroids	Presence (Light Green color)
Terpenoids	Salkowski test	Presence (Reddish brown color)
Monoterpenes	Test for monoterpenes	Presence (Red color)
Sesquiterpenes	Test for sesquiterpenes	Presence (Blue color)
Cardiac glycosides	Test for cardiac glycosides	Presence (A brown ring of the interface)

Phenols, Tannins and saponins in this herbal decoction were important due to antioxidant, anti-inflammatory, antibacterial, anti-asthmatic, and immunomodulatory actions (Lin et al. 2016). Steroids have antibacterial properties (Raquel, 2007) and due to their relationship with sex hormones they are very important compounds for gynecological disorders (Okwu, 2001). Phenols, Tannins and saponins in this herbal decoction were important due to antioxidant, anti-inflammatory, antibacterial, anti-asthmatic, and immunomodulatory actions (Lin et al. 2016). Steroids have antibacterial properties (Raquel, 2007) and due to their relationship with sex hormones they are very important compounds for gynecological disorders (Yadav and Agarwala, 2011; Okwu, 2001).

Total polyphenolic content was 3.38±0.07 mg gallic acid equivalents/ml of decoction and total flavonoid was not detected at 100, 500 and 1000 mg/ml of the herbal decoction. In general, medicinal plants which are rich in phenolic compounds have potential antioxidant properties (Brown and Rice-Evans, 1998; Krings and Berger, 2001; Ali et al., 2008; Molan et al., 2012; Huan xia et al., 2014). Insulin resistance is common in patients with PCOS. Insulin resistance can be improved through diet and lifestyle changes. Polyphenol-rich meals have been clinically demonstrated to improve insulin resistance (Lakshmi and Abirami, 2017). Polyphenol possesses anti-androgenic effects and inhibits the development of the dihydrotestosterone receptor complex. In addition, polyphenols inhibit testosterone hormone release (Williamson and Sheedy, 2020). According to previous studies *N. sativa* seeds and *S. indicum* seeds contain polyphenols and flavonoids, (Karunagoda et al., 2020; AL-Okaily et al., 2012; Dravie et al., 2020). At higher temperatures, flavonoids can be destroyed (Sharma et al., 2015). While preparing the decoction, both *N. sativa* seeds and *S. indicum* seeds were exposed to higher temperature for a prolong time. This may be the reason for not detecting flavonoids in the decoction. Present

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decoction exhibited dose dependent (R^2 = 0.979) scavenging activity towards 1-diphenyl-2-picryl hydrazyl (DPPH) radicals. IC_{50} value of the decoction was 850.5±30.2 µg/ml for the DPPH assay (Table 2 and Figure 2).

Table 2: Free radical (DPPH) scavenging activity of the decoction prepared with seeds of *Sesamum indicum* L. and *Nigella sativa* L.

Concentration (µg/ml) of the decoction	% Radical scavenging
400	26.77±1.79
600	35.60±2.57
800	43.00±1.70
1000	60.35±2.12
1200	70.67±1.92

Data represented as mean ± SE, extracts n=3

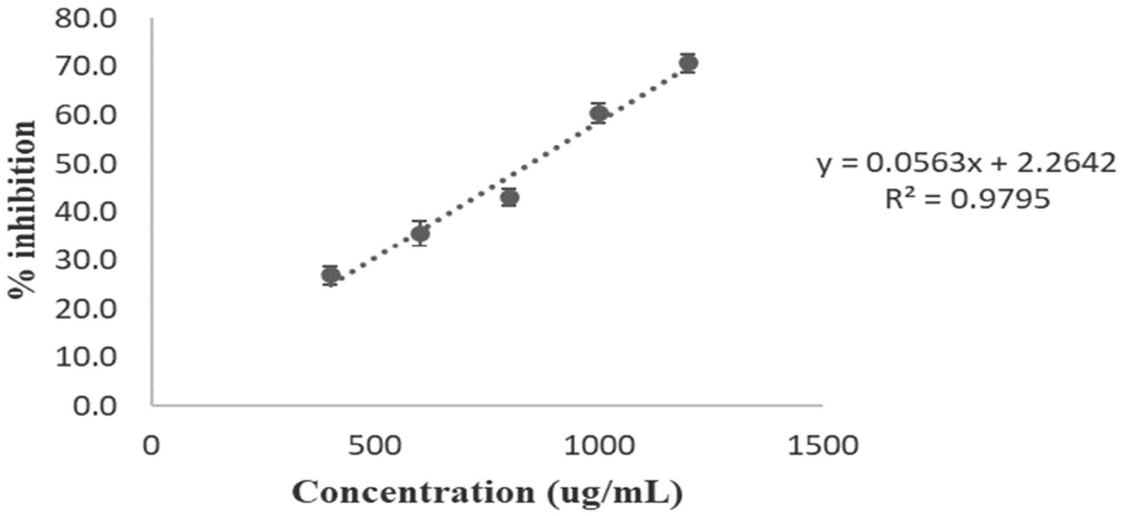

Figure 2. Dose response relationship of free radical (DPPH) scavenging activity of the decoction prepared with seeds of *Sesamum indicum* L. and *Nigella sativa* L.

It is proven that antioxidants have positive effects in management of PCOS and its' complications (Amini et al., 2015). *Escherichia coli* and *Coliforms* were not detected whereas Yeasts & Moulds and *Staphylococcus aureus* were present (less than 10 colony forming units (CFU) per 1 ml of the decoction). Microbial contaminations are most likely produced by inappropriate medicinal plants collecting, transportation, drying, preparation, storage, or dispensing practices (Lima et al., 2020). Good manufacturing processes in pharmacies and industries that handle herbal medications are critical, and they will help patients avoid unnecessary risks. Tested heavy metals such as As, Hg, Cd and Pb were not detected in the decoction. LOD (limit of detection) values for above heavy metals were 0.05 mg/kg. High quantities of potentially toxic heavy metals have been found in products available to the public in samples taken from both developed and developing countries (Awodele et al., 2013). Numerous

traditional remedies are known to cause significant acute renal pathology, the mechanism of which is yet unknown but has been linked to heavy metal poisoning (Awodele et al., 2013). Heavy metals are known to have limited renal clearance rates, even at very low concentrations, they may cause adverse effects in humans (Dghaim et al., 2015). Hence, quantitative determination of the presence of toxic metals is very much important. TLC fingerprint of the decoction revealed the presence of phytochemicals of both *N. sativa* seeds and *S. indicum* seeds (Figure 3).

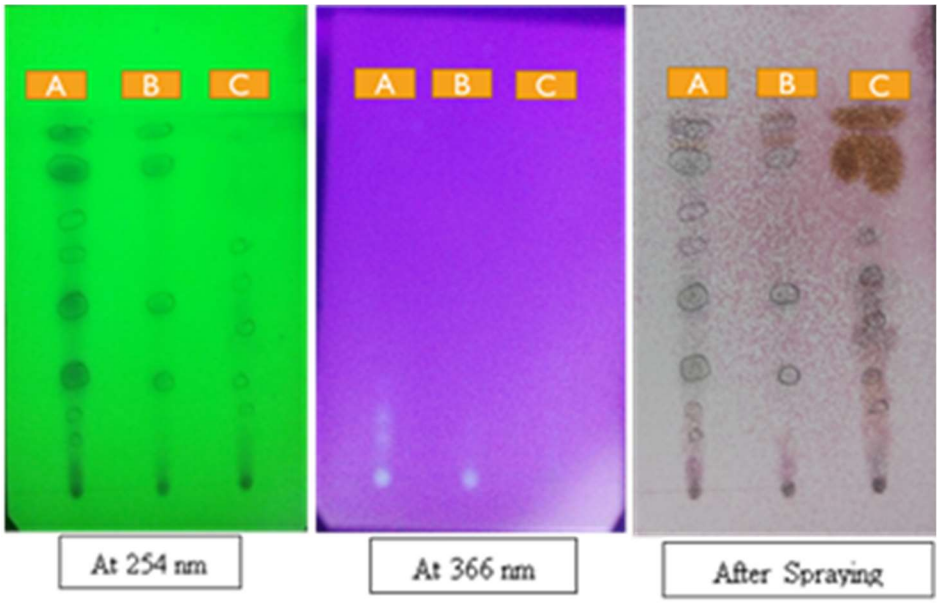

Figure 3. A- TLC fingerprint for *Nigella sativa* L, B- TLC Fingerprint for tested decoction (Prepared by *Nigella sativa* L and *Sesamum indicum* L) and TLC Fingerprint for *Sesamum indicum* L.

CONCLUSION

Nowadays, the use of antioxidants in the management of women with PCOS has attracted lots of interest. Some characteristics of PCOS such as obesity and abdominal adiposity, androgen excess, and insulin resistance can develop oxidative stress in these patients. Reported phytochemicals and antioxidant properties may be responsible for the remedy of PCOS while absence of harmful microbes and heavy metals are the indication of the safety of the decoction. These data can be utilized as quality control and quality assurance reference standard for the decoction in the future and can be utilized for Ayurveda pharmacopeial development process.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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