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Potential molecular ameliorating effect of *Calluna vulgaris* phenolic compounds on lipopolysaccharide-induced testicular inflammation

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VI. SUMMARY AND CONCLUSION

Infertility is defined as the inability to conceive after at least 1 year of regular, unprotected sexual intercourse. It affects 8-12% of couples globally in the reproductive age. In Egypt, the total fertility rate decline from 3.5 in 2014 into 3.1 in 2018, and in 2021 the current fertility rate is 3.211 births per woman, a 1.2% decline from 2020. The causes of male infertility are diverse, but can be related to congenital, acquired, or idiopathic factors that impair spermatogenesis. Lipopolysaccharide (LPS), major component of all Gram-negative bacterial cell wall, used to stimulate inflammatory response associated with cellular oxidative stress especially in mitochondrial rich organs as in testis.

Oxidative stress plays a centrals role in the pathophysiology of male infertility. Thus, it comes the term "Male Oxidative Stress Infertility" (MOSI); characterized by infertile men without a discernible cause, having abnormal semen characteristics alongside with oxidative stress. Traditionally, male oxidative stress infertility (MOSI) was treated by using antioxidant compounds such as fat-soluble vitamin E, β-carotene, coenzyme Q, or water-soluble vitamin C and GSH/selenium, or by terrestrial natural products which have antioxidant activity like *Maca*, *Ginseng* and *Pygeum*. Other promising natural product belongs to *Ericaceae* family is the *Calluna vulgaris* (Cv), that contains diverse classes of polyphenols which are the chief agents responsible for its biological function and curative effects.

In our previous study, the biological screening of two selected crude-ethanolic extracts of *Berberis* and *Calluna vulgaris* were analysis *in vitro* and *in vivo* studies. Interestingly, the collected data revealed that *Calluna vulgaris* showed higher prophylactic effect than *Berberis vulgaris* aganist LPS-induced hepatocellular toxicity. Therefore, in the current study, the crude -ethanolic extract of *Calluna vulgaris* (Cv) was selected for further investigations.

The aim of the present study is to improve the antioxidant/anti-inflammatory properties of *Calluna vulgaris* crude-extract via increasing the purification folds of polyphenol compounds, thus it can to be applied with lower dose and higher efficiency against acute or chronic testicular inflammation mediated male infertility. Also, to investigate the possible prophylactic/ therapeutic effects of phenolic compounds extracted from *C. vulgaris* crude. Therefore, comparative *in vivo* study was done between the Cv crude-ethanolic extract and its most active fraction obtained from the crude. Furthermore, aiming to understand the molecular pathways mediated male infertility, so multiple networks of testicular autophagy, apoptosis and oxidative stress or inflammation were studied.

Liquid-liquid extraction method was used to fractionate the Cv crude-ethanolic extract by successive solvent extractions, using petroleum ether, dichloromethane, ethyl acetate and with n- butanol fraction and finally the residual aqueous layer was collected.

Comparative *in vitro* studies were done between Cv crude- ethanolic extract and its five fractions by measuring the total phenolic content by Folin–Ciocalteu method, also polyphenol compounds of both Cv crude -ethanolic extract and Cv-ethyl acetate fraction were detected by high performance liquid chromatographic (HPLC) analysis by using seventeen different polyphenols as standards. Furthermore, the *in vitro* antioxidant capacity (DPPH and NO

scavenging activities) and the Human red blood corpuscles (HRBCs) membrane stabilization of Cv crude-ethanolic extract or its five fractions and *E. coli*-LPS were measured.

In order to induce testicular inflammation (acute/chronic response), lipopolysaccharide (LPS) was extracted from *E. coli* by methanol-chloroform extraction method. Two testicular inflammatory scheme models were applied as simulation and applicable to human infertility. The first one was performed by administrating single dose of lipopolysaccharide (LPS) to stimulate acute inflammatory response and the second LPS scheme was done by using multiple LPS doses to enhance chronic inflammatory response. Those were applied *in vivo* by using sexually mature seventy-two male rats which were equally categorized into 12 groups.

The sham group; comprised healthy individuals with normal seminal parameters, **vehicle group;** that received polyethylene glycol 400x (PEG400x) orally via gavage tube 20%v/v, 0.5 ml/daily for 4 weeks period , **Cv crude-ethanolic extract control group**; that taken tube Cv- EtOH extract at dose 200mg/kg/day orally by gavage for 4 weeks, **Cv – ethyl acetate fraction control group**; received orally by gavage tube Cv- ethyl acetate fraction at dose 57.14 mg/kg /day, **Single LPS induced group**; that administrate LPS injection 4mgI.P/Kg for 12 hours, the pretreatments group; **Cv crude-ethanolic extract pretreated group**; received Cv-EtOH orally by gavage tube at dose 200mg/kg/daily for 4 weeks, then single LPS injection at dose 4mg I.P/Kg for 12 hours and **C.v–ethyl acetate fraction pretreated group**; given Cv-EtOAc Fr. orally by gavage tube at dose 57.14mg/kg/day for 4 weeks, then single LPS injection (4mgI.P/Kg) for 12 hours.

For the multiple LPS admiration (chronic model) the groups were designated as follow; **2 weeks LPS-induced group;** those were injected with LPS at dose 250 µg IP /Kg/day after day for 2 weeks**, 4 weeks LPS-induced group**; received LPS at dose 250 µg IP /Kg /day after day for 1 month and finally the three treatments groups; **Treatment with vehicle group**; rats were intra-peritoneal (IP) injected with LPS at dose 250 µg /Kg body weight day after day for 2 weeks then given orally by gavage tube PEG400 (20%v/v, 0.5 ml/day) + LPS at dose 250 µg /IP /Kg /day after day for 2weeks and the last 2 weeks rats were orally given PEG400 (20%v/v, 0.5 ml/day), **Cv crude-ethanolic extract - treated group**; administrated LPS at dose 250 µg/ IP /Kg /day after day for 2 weeks then given orally by gavage tube Cv- EtOH extract (200 mg/kg/day) + LPS at dose 250 µg /IP /Kg /day after day for 2 weeks and the last 2 weeks rats were orally given Cv crude-ethanolic extract at dose of 200 mg/kg/ day and finally **Cv–ethyl acetate fraction treated group**; given LPS at 250 µg IP/Kg/day after day for 2 weeks then the next 2 weeks rats were given orally via gavage Cv-EtOAc Fr. at dose 57.14mg/kg/day + LPS at 250 µg /IP /Kg / day after day and the last 2 weeks rats were orally given via gavage Cv-EtOAc Fr. at dose 57.14 mg/kg/day.

The serum, epididymis and testes were collected from the entire study groups. Liver and kidney functions were determined in serum spectrophotometrically. Serum testosterone and prostatic specific antigen levels were determined by ELISA technique. Epididymal seminal parameters were assessed by CASA technique. In testicular homogenate, the oxidative stress indicators as pro-oxidant concentration including, NO, TBARS levels and XO specific activity were measured. Also, the enzymatic and non-enzymatic antioxidant profiles as GSH level, SOD and GST specific activity were detected spectrophotometrically. Additionally, autophagy flux levels as beclin-1 or mTOR were measured, also the apoptotic parameter as in p-p53 (ser15)/ p53 total ratio levels, and the

angiogenic marker (VEGF) and inflammatory marker (IL-1β) were measured by ELISA technique. The pro-inflammatory cytokines as TNF-α, ADAM-17 and iNOS were quantified in testicular gene expressions by using real time PCR technique. Furthermore, histological examination of testicular and epididymal tissues were performed.

The *in vitro* results revealed that, among the five fractions extracted from Cv crude-ethanolic extract, Cv- ethyl acetate fraction had the highest total polyphenols content where the purification fold was increased three times as compared to Cv crude -ethanolic extract. Therefore, the examined *in vivo* doses were 200 mg/kg for crude extract and 57.16 mg/kg for Cv -ethyl acetate. Although the two examined doses had the same total phenolic content, but we could minimize the applied dose of ethyl acetate fraction three times as compared to Cv -ethanolic crude extract. Moreover, the two examined extracts contain variable mixtures of phenolic compounds, confirmed by HPLC analysis where the distribution of phenolic compounds that were present in Cv crude-ethanolic extract were affected by fractionation when using ethyl acetate, where the most dominant hydroxy-cinnamic acids in Cv-ethyl acetate fraction was p-coumaric acid that increased by 9 purification fold. Among Hydroxy-benzoyic acids, that vanillic acid showed 19 purification fold while among flavonoids, Apigenin-7-glucoside was increased by 78.69 folds after purification. These positive increments might return to the ethyl acetate solvent polarity that extracted the semi polar compounds like polyphenols based on the rule of 'like and dislike property.

The *in vitro* antioxidant capacity (DPPH and NO scavenging activities) of Cv crude-ethanolic extract and its fractions showed that Cv-ethyl acetate fraction significantly had the most effective scavenging activity. Also, Cv- ethyl acetate fraction had the highest antioxidants properties, alongside with the highest hemolytic inhibitory effect on human red blood cells due to the close-relation between inflammatory and oxidative stress pathways

Depending on the *in vitro* studies that were performed in Cv crude –ethanolic extract and the five fraction extracted forms, results showed that both Cv- ethyl acetate and the crude one had the best antioxidants/anti-inflammatory and the highest phenolic content as well. Therefore, both of them were selected for the *in vivo* study.

The *in vivo* collected data showed that, both LPS schemes; the acute and chronic one had the same molecular adverse effect pathways. However, the chronic LPS administration showed more severe testicular inflammatory changes than the acute one. For the treatment strategies both the preventive/therapeutic properties found in Cv-ethyl acetate had higher efficacy than the Cv crude-ethanolic extract.

By single or multiple LPS administration, could induced systematic sepsis confirmed by multiple organs damage as in kidney and liver, evidenced by elevated ALT, AST activities that leaked from tissues into blood. Also, urea and uric acid were elevated. Another organ that was seriously affected by LPS administration was the male reproductive system. LPS induced testicular dysfunction through inducing inflammatory cascade reactions. In fact, begins with high flow of blood supply to the affected area, increase in capillary permeability as confirmed by high levels of VEGF in testicular homogenate to enable larger serum molecules to enter the tissues and finally increase the migration of circulating monocytes from blood to testicular tissues. Once monocytes enter the tissues, they are differentiated into macrophages, these recruited cells with testicular

germ cells as Sertoli cells and Leydig cells release numerous mediators as pro-inflammatory cytokines such as ADAM-17, TNF-α, IL-1β and iNOS in testis. The TNF-α, IL-1β are interfering with the normal process of male fertility through the inhibition of serum testosterone biosynthesis in LPS-induced groups. The recruited leukocytes to the testis increased reactive oxygen species (ROS) as well as reactive nitrogenous species (RNS), confirmed by high TBARS, NO levels and increased XO specific activities. Those increments had negative impact on sperm parameters it decreased both sperm count and motility that was assessed by CASA, via breaking the double bond in the polyunsaturated fatty acids in sperm cell membrane which in turns decreased membrane fluidity, essential for proper motility. Oxidative stress was not only confirmed by high free radicals but also by significant decreased in detoxification processes as the low level of GSH, and decreased in SOD and GST specific activities.

Another important factor that exaggerated the testicular oxidative damage was through the impaired autophagy, confirmed by decreased in beclin-1 level and increased in mTOR (autophagy inhibitor). Defective autophagy led to inability for ROS/damaged mitochondria clearance. Therefore, accumulation of damaged proteins that in fact promote aberrant activation of multiple signaling pathways culminating in apoptotic cell death. Also, p-p53 (ser15)/ p53 ratio was elevated by LPS administrations which might be due to the high level of mTOR kinase, which directly binds to p53 and phosphorylates it at serine 15 which in turn increased apoptosis/autophagy ratio levels.

Those molecular investigated deteriorated mechanisms were confirmed by histological investigations. LPS-induced groups (single/multiple doses) depressed spermatogenesis, distorted the seminiferous tubules as it showed necrotic spermatogonia along with degenerative germ cells and appearance of vacuoles observed in testicular section. Also, leukocytes infiltration, hemorrhage and edema were observed in epididymal section.

The two treatment strategies of Cv-ethyl acetate fraction; the preventive against single LPS/ acute inflammatory scheme and the curative against multiple LPS administration/chronic one had inhibitory effect towards ADAM-17, TNF-α, IL-1β and iNOS gene expressions in testicular homogenate, that might be due to Apigenin-7-glucoside which was high rich in Cv-ethyl acetae fraction and was reported to decrease those inflammatory cytokines. Also, Cv-ethyl acetate fraction decreased the protein levels of IL-1β and VEGF in testicular tissues.

Cv-ethyl acetate fraction ameliorated the LPS adverse effects via their antioxidant and free-radical scavenging properties, that confirmed by the elevation in enzymatic antioxidants activities as SOD and GST and non-enzymatic level of GSH as compared to induced groups. That subsequently normalized the pro-oxidant parameters as NO, TBARS levels as well as XO specific activities.

The administration of Cv-ethyl acetate fraction was able to activate autophagy flux confirmed by beclin 1 elevation and decreased both mTOR alongside suppression of apoptosis through p-p53 (ser15)/ p53 ratio level. Activated autophagy could clear all damaged organelles in testis and also participate in testosterone biosynthesis, spermatogenesis, suggesting that polyphenols not only act as antioxidants and inflammatory cytokines augmenters but also as autophagy activators.

Additionally, Cv-ethyl acetate fraction showed fertility enhancing effects that were confirmed by elevation in serum testosterone level and reduction prostate specific antigen (PSA) as compared to LPS- induced group. Also, its increased sperm count and motility thus full restoration of testicular function and fertility preservation has been revealed.

The synergetic effects of different classes of concentrated polyphenols as flavonoids purified may influence the production of androgens in Leydig cells as they share structural similarity between cholesterol and other steroids, which are consequently associated with increase in sperm count.

The histological examinations of Cv-ethyl acetate fraction could restore and protected the testicular and epididymal tissues. It showed complete regenerative of seminiferous tubules, near-normal spermatogenesis with sperm formation in the testicular sections and little mild interstitial inflammation in the epididymal sections were observed in Cv-ethyl acetate groups. Altogether, data showed that ethyl acetate fraction seems to have more potent effect than that of Cv crude–ethanolic extract.

Conclusion

- The administration of LPS induced male infertility through activation of pro-inflammatory cytokines from migrated leucocytes or immature spermatozoa which in turn lead to oxidative stress propagation.
- Increased apoptosis/ autophagy ratio in LPS induced group due to oxidative stress/ inflammatory axis that activated apoptotic parameter and also stimulates mTOR signaling pathway which subsequently impaired autophagy.
- Testicular senescence phenomena caused spermatogenesis arrest and impairment, low sperm quality and testosterone synthesis reduction which finally resulted in male in fertility.
- The extract fractionation successfully increased the preventive/therapeutic potency of plant extract as the applied dose was decreased three times in case of Cv fraction when compared to its crude extract.
- Cv-ethyl acetate ameliorated tissue damage induced by LPS through increasing antioxidant levels, autophagy process and declining the pro-inflammatory and pro-oxidant levels which finally enhancing sperm quality and testosterone synthesis thus it can reverse male infertility.
- This plant is one of the plausible natural antioxidants that could be used as a lead candidate for synthesizing antioxidant drugs which can be used for the treatment of many oxidative stress related diseases.

Recommendations

- Further, validation and standardization of extraction method from collected plant from different region and time must be carried out.
- Complete UPLC/MS/MS and GC/MS must be done to have phyto-finger print for this fraction.
- Complete preclinical study that contained toxicity and pharmacokinetics must be carried out to measure toxic dose and understand the behavior of fraction in body organs on different animal models.
- Oxidative stress alongside with autophagy flux parameters should be considered as diagnostic parameters as well as during treatment in male oxidative stress infertility.