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EVALUATION OF PHYTOCHEMICAL ANALYSIS AND *IN-VITRO* EFFECT OF *TRIBULUS TERRESTRIS EXTRACT* ON SEMEN QUALITY

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ABSTRACT
Approximately half of human infertility cases are attributed to male infertility and natural herbs are promising medicines used to cure infertility. Worldwide, it is accepted that over 80% of the population of developing countries directly or indirectly relies on herbal medicine. This study was conducted to check the phytochemical analysis and effect of *Tribulus terrestris* extract on semen quality. A preliminary qualitative phytochemical analysis and quantitative analysis were performed to determine total phenolic contents (TPC) and total flavonoids contents (TFC) after ethanolic extract preparation of *T. terrestris*. The antioxidant potential of the *T. terrestris* sample was determined by DPPH radical scavenging assay. In-vitro effects of *T. terrestris* on sperm quality were also evaluated. A preliminary phytochemical analysis revealed bioactive compounds like glycosides, tannins, alkaloids, flavonoids, terpenoids, and quinones. Moreover, significant TPC and TFC were also found. Remarkable positive changes in sperm quality like motility and viability were observed after incubation with *T. terrestris* extract (50 ug/ml) for 60 to 120 minutes as compared to control group. *T terrestris* could affect male fertility capacity and this research could be a big success in curing male infertility using alternative herbal medicine without having side effects and being cost effective.

Keywords: Male infertility, Herbal Medicine, *T. terrestris*, Anti-oxidant potential, Sperm motility

INTRODUCTION
Maintenance of species is the dogma of all existing living organisms. All living organism try to maintain their species through the natural process of reproduction that is the major process, which enable them to represent itself in upcoming generation in the form of new off spring. Sexual interaction is one of the most significant social and biological relationships in human life (Montorsi et al., 2003). Male fertility can be defined as 'reproductive capability of a male that contain normal sperm quality and sufficient number with ability to mate his partner' (Oyeyem et al., 2008). Primary infertility is defined as 'Inability to conceive after one year of un protected sexual intercourse with her partner'. According to WHO 60 to 80 million people are infertile whole over the world. Pakistan is six most papules country in the world. It has been 182 million people and it also 21.9% infertility rate (Tahir et al., 2004). Many factors are involved in the process of conception that

affects both men and women, whereas 40% to 50% of infertility cases are the results of male infertility

Different modalities like treatment of follicle-stimulating hormone (FSH) and Human chorionic gonadotropin (hCG) are used to control male infertility. But these strategies are may be acceptability, surgical and cost issue (Walschaerts et al., 2013). Furthermore, offspring conceived through assisted reproductive technologies (ART), such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) and microsurgical epididymal sperm aspiration (MESA) exhibited an elevated risk of birth defects raising concerns (Egan et al., 2020). Furthermore, medicinal plants are widely employed by millions of individuals worldwide for addressing male infertility. The appeal of these remedies lies in their perceived safety and affordability, making them more attractive to people seeking treatment (Hussain et al., 2018).

The previous literature enlists the wide range of therapeutic effects like antioxidant, anti-inflammatory, antiulcerogenic, anticonvulsant, antidiabetic, cardioprotective, nephroprotective, nootropic, neuroprotective, and restorative properties. Potential herbal sources are also used to generate revenue worldwide, Recently, medicinal plants demand is increasing compared to allopathic medication due to various factors like broad range of therapeutic potential, safety and cost issue. Pakistan is also rich source of medicinal plants and nearly 600 to 700 medicinal species are documented (Hamad et al., 2023). Recently, beneficial effects of herbal medicine have been gradually increasing in developing countries and for male infertility numerous medicinal plants have been investigated. Use of herbal plants as antioxidants also getting attention and several medicinal plants like *T. terrestris* have high antioxidant potential (Khaleghi et al., 2017).

Mainly single plants are used and studied extensively. Previous literature was also documented *Withania somnifera, Crocus sativus*and *Tribulus terrestris* the aphrodisiac effects and promote the sperm count in male (Munier et al., 2022). *Tribulus terrestris* L belongings to Zygophyllaceae family and contains cluster of several biologically active compounds such as steroids, saponins, flavonoids, alkaloids, unsaturated fatty acids, vitamins and tannins. Various studies have been proven the spermatogenesis and libido effects of *T. terrestris* in in human and animals (Khaleghi et al., 2017; Grigorova et al., 2008). The main objectives of current research study were to find the effects of *T. terrestris* on male sperm count and overall sperm health.

MATERIALS AND METHODS

Plant Material and Preparations

Selected medicinal plant *T. terrestris* were procured from the local market of Faisalabad-Pakistan. *T. terrestris* was taxonomically identified and authenticated from Department of Botany, Government College University, Faisalabad Pakistan.

Plant material collection and processing

For extract preparation *T. terrestris* were grinded into powder. Powder (10g) were soaked into 100 ml ethanol solvents for 3 days with intermitted shaking at room temperature. Then placed it in shaking incubator for 3 days for further facilitation of evaporation. Filtrate was obtained using the Whatman filter paper. ethanol extract was dry in rotary evaporator under reduced pressure (Umbreen et al., 2024). The thickened extract was stored in a refrigerator for further use.

Phytochemical Evaluation

A preliminary qualitative phytochemical analysis was performed of *T. terrestris* for the identification of bioactive compounds like glycosides, tannins, alkaloids, flavonoids, terpenoids, and quinones using the standard protocols (Swargiary et al., 2021).

Quantitative Phytochemical Evaluation of *T. terrestris*.

After preliminary qualitative phytochemical analysis, quantitative phytochemical evaluation of *T. terrestris* like total phenolic contents and flavonoids were determined.

Determination of Total Phenolic Contents (TPC)

The total phenolic contents in sample was determined by Folin-Ciocalteu method as described by Naz *et al*. (2016). The calibration curve was prepared with different concentrations of gallic acid. 1mL aliquots of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.10mg/mL gallic acid solution in methanol were mixed with 5mL of Folin-Ciocalteu reagent (diluted ten folds) and 4mL of sodium carbonate (20%). The absorbance was noted after 1 hour at 765nm and the calibration curve was plotted by taking absorbance as a function of concentration. 1mL of sample extract (0.001g/mL) was mixed with the same reagent as described above and after 1 hour the absorbance of the resulting blue color complex was measured at 765nm. All determinations were performed in triplicate. Quantification was done with respect to the standard (gallic acid) (Sharif *et al*., 2018). Total content of phenolic compounds in plant extracts in gallic acid equivalents (GAE) were calculated by the following formula.

T = C x V / M

Where,

T = total contents of phenolic compound in mg GAE/g plant extract.
C = the concentration of gallic acid calculated from calibration curve in mg/mL.
V = the volume of extract in mL.
M = the weight of plants extract in grams.

Total Flavonoids Contents (TFC)

The total flavonoid contents of sample were determined according to the method given by Rehman *et al*. (2013). Briefly 0.5mL of sample was mixed with 2mL of distilled water and 0.15mL of 5% $NaNO_2$ solution and incubated for 6 minutes. After that 0.15mL of 10% $AlCl_3$ solution was added to that and again incubated for 6 minutes followed by the addition of 4% NaOH solution to the mixture. Volume of the reaction mixture was made up to 5mL by the addition of methanol and mix well. Absorbance of the reaction mixture was taken at 510nm after incubation for 15minutes (Ayub *et al*., 2017). Total flavonoid contents (TFC) of the extracts were expressed as µg catechin equivalents per mL of plants extract from the linear regression curve of catechin.

Evaluation of Anti-Oxidant Activity Using DPPH Scavenging Activity:

Antioxidant Potential of sample was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as described by (Shahid et al. 2014). According to this method 1mL of 0.004% DPPH in methanol solution (freshly prepared) was added to 3mL of *T. terrestris* extract sample and then mixture solution was kept for 30 minutes in dark. Then absorbance was noted at 517 nm. A low absorbance of reaction mixture indicates a high radical scavenging activity. The antioxidant activity of ascorbic acid as standard was also analyzed. The solution without plants extract was used as control (Naseem et al., 2020). All the tests were performed in triplicate. The percentage inhibition of DPPH radical samples was calculated as follows.

$$\text{DPPH Inhibition (\%)} = \frac{\text{Blank absorbance } (A_0) - \text{Sample absorbance } (A_1)}{\text{Blank absorbance } (A_0)} * 100$$

Where

A_1 = Absorbance of sample.
A_0 = Absorbance of blank.

In vitro spermatozoa parameters

Semen Samples Collection

Healthy individuals were selected semen collection and semen was collected through masturbation into a sterile container following a 3-day period of abstinence. The samples were then maintained at 37°C until liquefaction occurred, with non-liquefied samples monitored at 20-minute intervals until liquefaction was achieved. Microscopic analysis was carried out in accordance with the World

Health Organization (WHO) manual. All standard semen parameters fell within the normal ranges as outlined by WHO guidelines (Petyim et al., 2014).

Incubation of Spermatozoa with *T. terrestris* Extract

After sperm dilution and processing, the sperm sample were divided into 2 groups. Control group only provided with normal saline and experimental group were given with *T. terrestris* extract at doses of 50 µg/Ml. The parameters were measured after 30, 60, 90 and 120 minutes of incubation at 37°C. Semen quality analysis was performed using the computer-assisted semen analysis (CASA) version 12 IVOS. After 0 seconds and in every 30 minutes of incubation, the sperm motility parameters were observed using the CASA system.

Statistical Analysis

The results of experiments are expressed using mean + standard error of mean. Differences between two groups were compared by using Student's t test, and statistical comparisons between experimental groups were made by analysis of variance and Tukey's post test, as offered by GraphPadInStat version 3.0 (GraphPad Software Inc, La Jolla, CA). Statistical significance was determined as $P < .05$.

RESULTS

Phytochemical screening Phytochemical screening of TLM demonstrated the presence of alkaloids, anthraquinones, cardiac glycosides, coumarins, flavonoids, saponins, phlobatannins, tannins and terpenoids.

Determination of Total Phenolic Contents (TPC)

Significant amount of total phenolic contents was found in the extract of *T. terrestris* extract as shown in figure 1. The drawn standard curve was used for the concentration and absorbance of Gallic acid to calculate the total phenolic contents of *T. terrestris*.

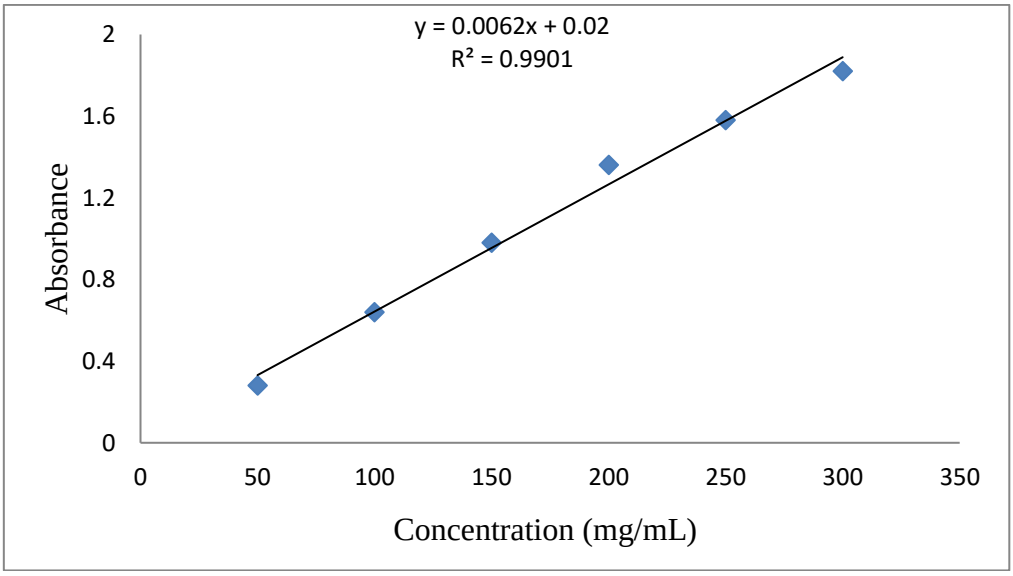

Figure-1: Standard curve of Gallic acid for TPC and dot represents the phenolic contents found in ethanolic extract of *T. terrestris*.

Determination of Total Flavonoid Contents (TFC):

The regression equation of calibration curve (y = 0.0034x; R2 = 0.9897) and expressed as mg quercetin equivalent (QE) per gram extract (mg/g) was used for determination of Total Flavonoids content of the extracts *T. terrestris*as shown in figure 2.

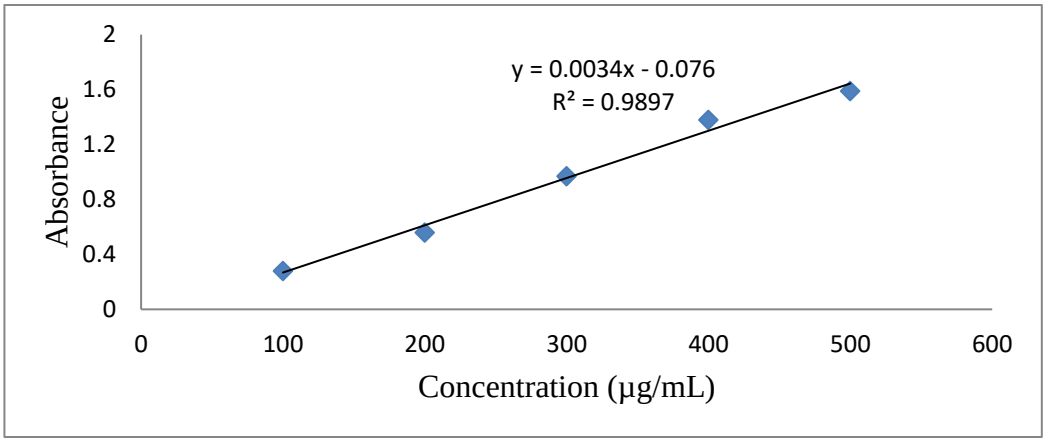

Figure 2: Standard curve of quercetin for flavonoids represents the flavonoid contents found in ethanolic extract of *T. terrestris*.

Evaluation of Anti-Oxidant Activity

DPPH radical scavenging activity Figure 3 shows that the scavenging effects of samples on DPPH radical and were in the following order: TLB > TLE > TLC > TLM >TLA > TLH. The EC50 values of scavenging DPPH radicals for the TLB and TLE were 41.0±1 and 62.0±2 µg/ml, respectively. Though the antioxidant potential of fractions was found to be low ($P < 0.05$) than those of ascorbic acid, the study revealed that TLB and TLE have prominent antioxidant activity; the presence of phenolic compounds (containing phenolic hydroxyls) are mainly found in these two fractions and could be attributable to the observed high antiradical properties of these fractions.

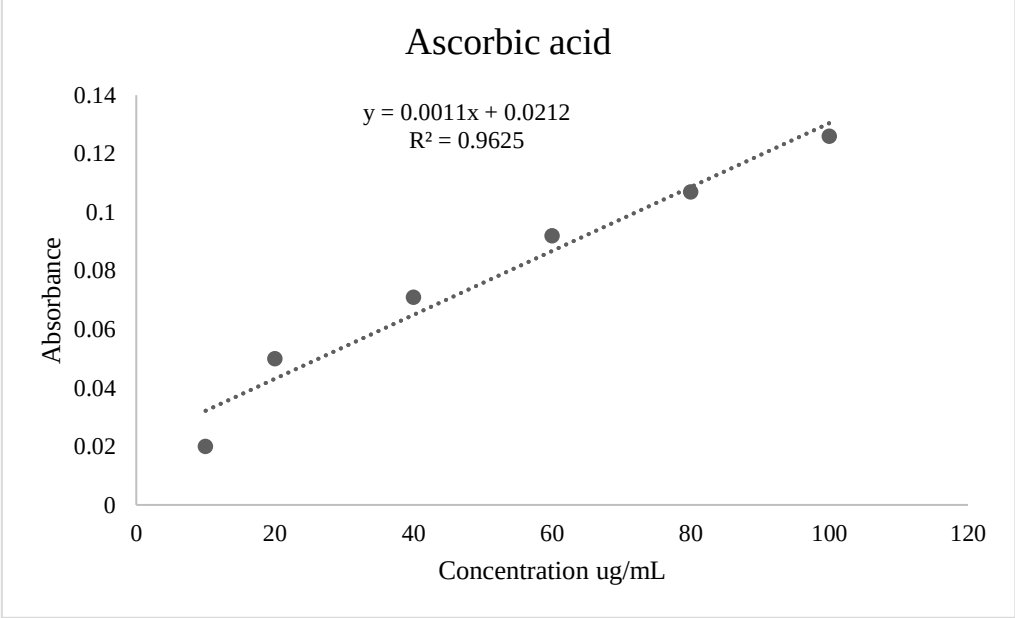

Figure 3: Evaluation of Anti-Oxidant Activity

In vitro spermatozoa parameters

In vitro spermatozoa parameters results revealed that there were significant ($p<0.05$) difference was observed between control and experimental group.*T. terrestris*treated semen represented the increased sperm motility as compared to normal saline treated semen at different time intervals like 30, 60, 90 and 120 minutes as shown in figure 4. Incubation of the semen with the extracts of *T. terrestris*improved the overall health of sperm like motility and preserved the viability of spermatozoa.

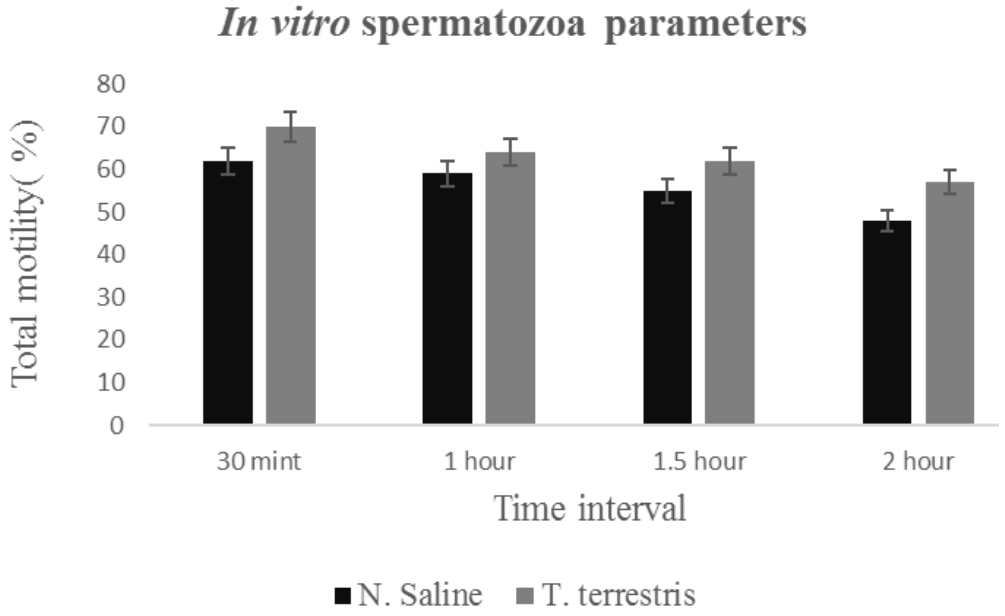

Figure 4: Effect of *T. terrestris* extract on the sperm motility.

Discussion

Herbal extracts are commonly used for treatment of infertility in male and the role of different plants well documented to improve the male health in context of infertility. This study focused on *T. terrestris* because it emerged as a new source of antioxidant for infertility therapy (Kumari et al., 2018).For centuries, various herbs have been employed globally as aphrodisiacs. The concept of a natural substance that can enhance libido and erectile function has captivated humanity. Among these herbs, *T. terrestris* has garnered attention due to its demonstrated ability to boost testosterone levels, lending credence to its aphrodisiac properties (Da Silva et al., 2024).

The findings of the current study suggest that *T. terrestris* extract significantly influences the motility and viability of human sperm. The ethanolic extract of *T. terrestris* is purported to elevate the body's natural testosterone levels, which positively impacts sperm qualities. Consequently, investigations revealed the fertility-enhancing potential of the plant's fruits. All gathered data supported increased sperm motility counts, elevated testosterone levels, and enhanced ascorbic acid levels. Moreover, the notable weight gain observed in all reproductive organs may be attributed to heightened androgen biosynthesis, leading to an increase in serum testosterone levels.

Present motility results are also correlated by another previous study which was conducted by (Mohaisen et al 2015) was use following herbal combination *T.terrestris*, *Phoenix dactylifera* and *Nasturtium officinale* to improve the semen quality and sperm motility in mature male albino mice. Different groups were treated with alternate doses with this herbal combination and in the results significant increase the sperm concentration and motility of the sperms in all treated groups.

(Zena et al 2023) conducted research on the impact of *Eruca Sativa* leaf extract on sperm parameters and testosterone levels. Their study involved 24 mice divided into four groups, with two groups receiving treatment doses of 30 and 40 mg/kg body weight. The results showed a significant increase in all parameters, particularly serum testosterone levels and sperm motility, upon administration of Sativa extract. Similarly, (Walid et al 2021) conducted a study to investigate the effects of *T. alatus* extract on the testosterone levels of male mice. Some past studies also conducted for evaluation of toxicity effects of *T. terrestris* with different doses. No histopathological changes were observed in the liver, spleen, kidney, and testis of all animals, and there were no abnormalities in causality or hematology. (Abdullaev et al., 2003). A maximum dose of 500mg/kg was administered to evaluate the oral toxicity of the drug, resulting in a significant improvement in sperm motility and reproductive capacity. All of the aforementioned studies demonstrate that herbs are relatively safer compared to synthetic drugs.

Conclusion

The present study concludes that *T. terrestris* is effective to increase the sperm motility, sperm count and testosterone level. *T. terrestris* extract contained significantly TPC and TFC with good anti-oxidant potential. Furthermore, the use of *T. terrestris* did not compromise the safety level and hence can be use in human to treat infertility.

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Declaration of Competing Interest

The authors declared that they don't have any financial interest respect to the content of this research

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