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RESEARCH ARTICLE

Evaluation of the Effects of Different Solvents Extracts of Tribulus Terrestris Obtained Via Ultrasonic Extraction on Human Asthenozoosperm Activation *In Vitro*

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Abstract

Objective: This study was designed to evaluate the activity of different fractions of Tribulus terrestris *Tt* obtained via 20KHZ ultrasonic technique for different periods of extraction time and different solvents on different asthenozoospermic parameters *in vitro*. Material and method: The study was conducted on seventy-five asthenozoosperic patients. The sperm samples were activated *in vitro* (after liquefaction) with different doses of the extracts by using a direct simple layer method (DSLM) technique. Grades of sperm movement and sperm morphology were the main parameters tested. Results: Aqueous extract for 15 min. at a dose 0.02% showed the highest activity. Less activity was obtained by exposure of the sperm samples to a dose 0.002% aqueous acetonitrile (AA) extract for 15 min., 0.0002% dose of Ethanol (EtOH) and Methanol (MeOH) extracts for 15 min., 0.002% dose of (AA) extract for 15 min. and 0.002% dose of (EtOH) and (MeOH) extracts for 60 min. Exposure of asthenozoospermic samples to a dose of 0.0002% of (AA) extract for 60 min. gave the lowest activity. Conclusion: These finding indicated that the use of ultrasonically assisted extraction can obviously improve the activity of *Tt* extracts on asthenozoospermic patient's parameters including the grades of sperm motility and sperm morphology.

Keywords: *Asthenozoospermia; Ethanol (EtOH)*; *In vitro activation; Methanol (MeOH); Tribulus terrestris Tt; ultrasound extraction*.

Introduction

Although nowadays there is still some debate that in a period that medicinal drugs still represent the main source for the basic health requirement in the industrial countries and that the use of medicinal plants has increased in the developed countries in the last decades [1]. Herbal remedies are prescribed widely after the increasing recognition of these products, their effectiveness, with a lower side effect than synthetic drugs and relatively less cost [2-4]. *Tribulus terrestris* L. *"Tt"* (Puncture Vine) belongs to the Zygophyllaceous family. It is a widely distributed in many regions all over the world such as Mediterranean, desert environment and subtropical regions. The different activities of the whole plant were investigated, the phytochemical, pharmacological, aphrodisiac, antidiabetic, analgesic, anti-inflammatory, hepatoprotective, anticancer, antibacterial and antifungal [5, 8] and effects on the female reproductive system[9]. A lot of compounds were reported to be present in this plant, namely saponin, alkaloids, amides and flavonoids [10, 11]. Many studies demonstrated that the saponin compound termed protodioscin is one of the compounds that was claimed to be responsible for many of the biological activities of *Tt* extracts [12, 13].Many methods were investigated to obtain the active an ingredient from medicinal plants among which is the use of sonication extraction. The enhancement of ultrasonic extraction can be explained as a dramatic change in the cells including cell disruption, changes in the permeability of the cell membrane which resulted in the release

of cellular constituents in to the surrounding medium. The use of ultrasonic extraction depends mainly on the used solvent [16]. The use of water in ultrasonic extraction in certain instances exhibited a lesser yield of active ingredients than organic solvents which produce less free radical than water using the same ultrasonic extraction procedures.

On the other hand, the use of ethanol in ultrasonic extraction seems to enhance the yields greatly since this solvent is a poor conventional solvent [17]. It has been reported that asthenozoospermia is among the common causes of infertility. This defect is characterized by a reduction in the movement or absence of the sperm motility in the newly ejaculated specimen. It is well known that motility of sperm in natural maturation is started when these sperms transit through epididymis. The motility of sperm is very necessary for the movement of these sperm from the vagina toward the fallopian tube to penetrate the cumulus oophorus and induce fertilization [18]. Although the etiology of asthenozoospermia still not explained precisely [19], a natural change in the internal and external factors responsible for the regulation of sperm motility and hence cellular metabolism and structure may be responsible for induction of tail beat which in turn could induce sperm motion abnormality leading to infertility [20].

The decreased success in the different treatment methods of male infertility gave the assisted reproductive technology, including the use of artificial insemination by the husband after the enhancement of sperm function in vitro, an important role in male infertility. This investigation was conducted to evaluate the activity of *Tt* extracts obtained by 20KHZ sonication with different solvents and different periods of extraction on certain asthenozoospermic parameters in vitro.

Materials and Methods

Plant Materials

The plant material was obtained and authenticated by the Department of Herbal Medicine/ Health Ministry, Iraq. The aerial parts of *Tt* were air dried, powdered to the required size through a standard mesh sieve No. 10.

Sample Preparation for Ultrasonic Extraction

The finely powdered aerial parts plant material (30g samples) were extracted separately two times with 300 ml of deionized water, 50% AA, 80% EtOH and 80% MeOH by sonication with 20KHZ frequency *(Model: VOX 600, Serial No. 30005F, Sonilab System UK)* for 15 and 60 min. at a temperature range of 23-27ºC.The extract of each solvent and period were filtered evaporated by using a rotary evaporator and added together, freezed to -70C, lyophilized using a freeze dryer and the dry weight was recorded. All extractions were done in three replicates, the percentage yields are illustrated in Table 1.

Preparation of the Different Dose of Plant Extracts

A stock solution of 2% was prepared from each plant's extract obtained after extraction with H_2O, 50% AA, 80% EtOH and 80% MeOH for the two periods of time (15 and 60min.). Then ten- fold serial dilutions were performed to prepare doses of 0.2, 0.02, 0.002 and 0.0002% from each extract to be checked for *in vitro* activation on the asthenozoospermic samples.

Seminal Fluid Analysis

Seventy five patients attending the High Institute for Infertility Diagnosis and ART, Al-Nahrain University, Baghdad, Iraq, with asthenozoospermia were involved in this study. The average ages of the patients were 25-50 years. The seminal fluid specimens were collected from the patients 3-5 days of abstinence directly in a clean, dry and sterile plastic container after masturbation in a private room adjacent to the laboratory of the seminal fluid analysis. The specimens were incubated in a 5% CO2 incubator at 37C for a period of 30 minutes to allow the semen liquefaction [21].A direct simple layer method (DSLM) without centrifugation [22].Was used in which the liquefied semen was carefully mixed for few seconds, and then one ml of the specimen was placed at a round bottom test tube, over layered by one ml of the selected dose of the sonicated *Tribulus terrestris* extract with pH adjusted to 8.0-8.2 (since this is the suitable pH for sperm motility [23], and then the specimen incubated in a 5% CO2 incubator at 37C and 90% humidity for 30 minutes. Then, one drop (approximately, 10μl) of the remaining liquefied semen sample (used as a control) and the semen

sample after activation with the sonicated plant extract were examined microscopically to check the different sperm parameters namely: sperm concentration, sperm motility "%" (progressive sperm motility "grade A (GA)", non-linear motility "grade B (GB)" and normal sperm morphology (%). Criteria for normal semen values were applied for evaluation of the results [24].

Statistical Analysis

Crude data were collected and analyses using Graph Pad Prism 7.0a through two ways analysis of variance (ANOVA) multiple comparison involving the mean and standard error of the mean (SEM) to detect the significant differences between the liquefied and non-liquefied samples (pre and post activation) of the groups.

The value was considered statistically significant when the *P* value less than 0.05.

Results

The activity of the different doses (2%, 0.2%, 0.02, 0.002% and 0.0002%) of each solvent (H_2O, 50% AA, 80% EtOH and 80% MeOH) extracted for 15 and 60min. using a 20KHZ sonication, on semen samples *in vitro* were tested to choose the most effective doses.

20KHZ Ultrasonic Extraction for 15min

Effect of H_2O, 15min. Extraction (0.02%)

The effect of 0.02% dose of H_2O *Tt* extract obtained after 15min. extraction on sperm parameters obtained from eight patients is presented in Figure 1. A. A significant ($P< 0.05$) increase (from 5.62% before activation to 22.5% after activation) in the GA% was found after incubation of the sperm samples with the H_2O extract.

The increase in the nonlinear (GB) % was found to be from 40% before activation to 46.88% after activation. The sum of the progressive and nonlinear sperm motility percentage (GA +GB) was increased from 45.63% to 69.38%.On the other hand, a significant increase ($P<0.05$) was found in the normal sperm morphology before activation (33.75%) compared to that after activation (75.63%). The normal sperm morphology was also increased significantly ($P<0.05$) and it was found to be 33.12%.

Table 1: The percentage yields by the ultrasonic extraction.

Solvents	% at 15min. extraction period	% at 60min. extraction period
H2O	11.40%.	14.60%.
50% aqueous acetonitrile	14.03%.	17.03%.
80% EtOH	12.10%.	13.56%.
80%MeOH	11.30%.	12.40%.

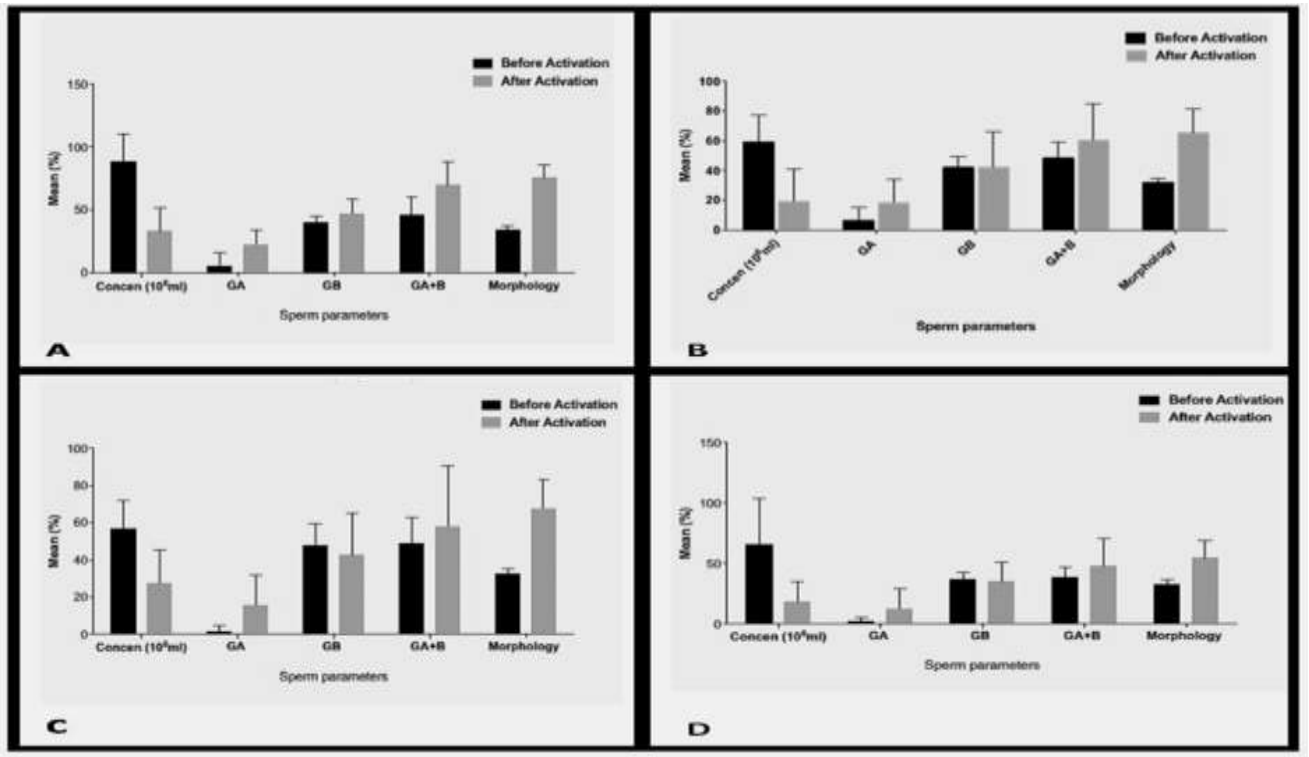

Figure 1: Effect of different solvent extract of *Tribulus terrestris* obtained via 20KHZ extraction for 15min. on asthenozoospermic parameters *in vitro*. A: Effect of H_2O extract (0.02%), n = 8. B: Effect of 50% aqueous acetonitrile extract (0.002%), n = 8. C: Effect of 80% EtOH extract (0.0002), n = 8. D: Effect of 80% MeOH extract (0.0002), n = 12

Effect of 50% AA, 15 min. Extraction (0.002%)

Figure1. B. illustrates the effect of 50% aqueous acetonitrile *Tt* extract at a dose of 0.002% on the different sperm parameters of eight patients. These results demonstrated that activation of the asthenozoospermic sperm samples with this dose increased the progressive sperm motility GA from 6.25% before activation to 18.13% after activation. The linear sperm motility was not affected after activation (41.88%) compared to that before activation (41.88%). The sum of GA+GB was increased from 48.13% before activation to 60% after activation. The normal sperm morphology was also increased significantly (P<0.05) and it was found to be 33.12%.

Effect of 80% EtOH, 15min. Extraction (0.0002%)

Figure1.C. shows the effect of 80% EtOH of *Tt* extract activation at the dose 0.0002% on asthenozoosperic patient's parameters (eight) *in vitro.* The increase in the percentage of forward progressive movements of sperm after activation for 30min. *in vitro* with a dose 0.002% was 5.62%, whereas, no difference was obtained at GB percentage of sperm motility between before and after activation with *Tt* extract. The total percentage of progressive sperm motility and linear motility was increased from 39.63% before activation to 44.38% after activation. The normal morphology percentage of sperms was obviously increased from 29.38 before activation to 45.63% after activation.

Effect of 80% MeOH, 15min. Extraction (0.0002%)

The results are presented in Figure 1. D. Activation of asthenozoospermic samples (twelve patients) *in vitro* with this extract revealed that the GA% was increased from 2.08% before activation to 12.50 % after activation. The GB% parameter showed no difference before (36.75%) and after (35%) activation with this extract at the mentioned dose. The sum of GA% and GB% of sperm motility showed an increase of 8.67% after activation. The normal morphology percentage of sperms increased significantly (P< 0.05) from 32.5% before activation to 54.58% after activation.

20KHZ Ultrasonic Extraction for 60min

Effect of 50% AA, 60min. Extraction (0.002%)

The effect of this solvent *Tt* extract at a dose 0.002% obtained after 60min. extraction on eleven asthenozoospermic patients is presented in Figure 2. A. The increasing in the GA% after activation for 30min. was significant (P< 0.05), in which it increased from 3.64% before activation to 19.55% after activation. The sum of GA% + GB% was not affected by the *Tt* extract activation. The percentage of normal morphology of sperm was significantly increased (P< 0.05) from 35.91% before activation to 68.64% after activation.

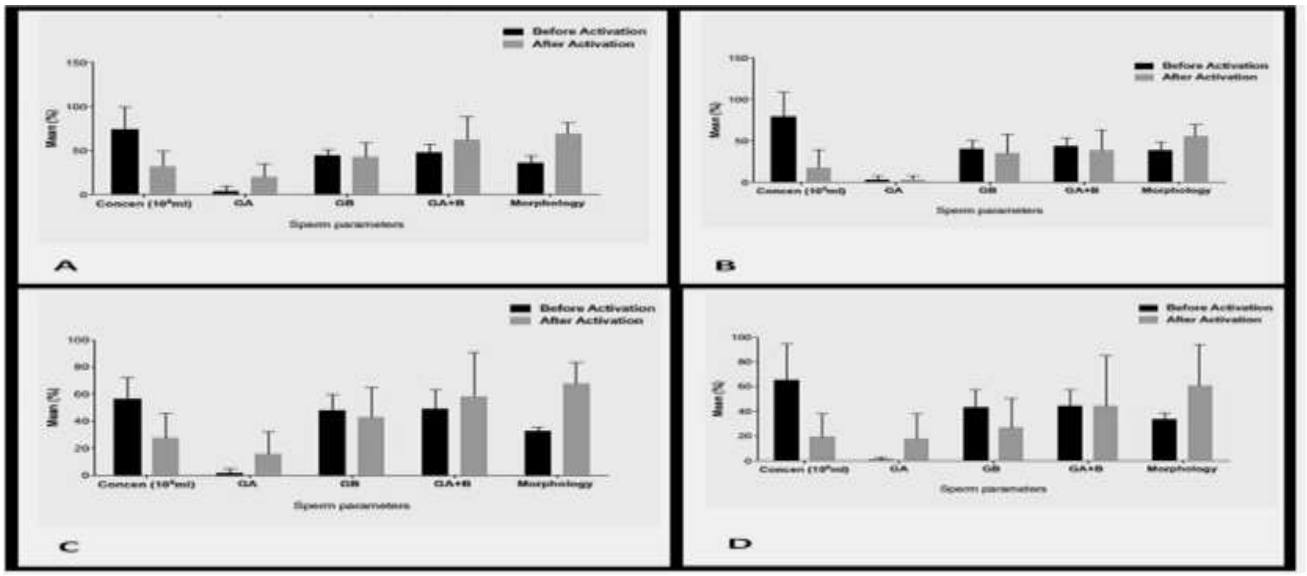

Figure 2: Effect of different solvent extract of *Tribulus terrestris* obtained via 20KHZ extraction for 60min. on asthenozoospermic parameters *in vitro*. A: Effect of 50% aqueous acetonitrile extract (0.002), n = 11. B: Effect of 50% aqueous acetonitrile extract (0.0002%), n = 8. C: Effect of 80% EtOH extract (0.002), n = 8. D: Effect of 80% MeOH extract (0.002%), n = 12

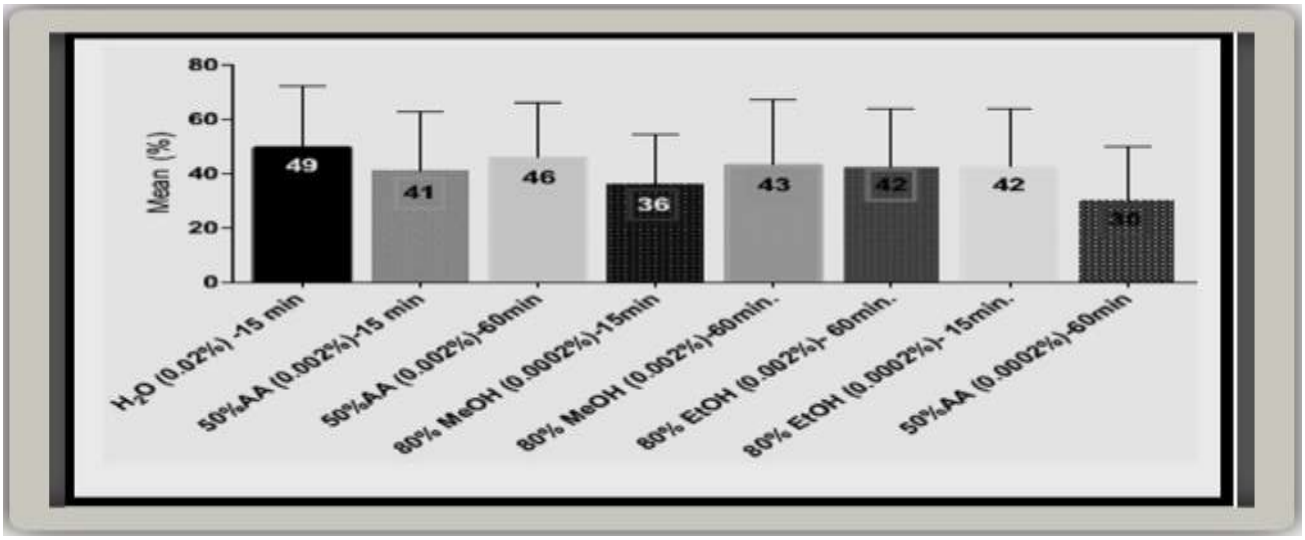

Figure 3: Comparison of the activity of the different extracts of *Tribulus terrestris* at different dose levels on the asthenozoospermic patient's parameters *in vitro*

Effect of 50% AA, 60min. Extraction (0.0002%)

The effect of 60min. *Tt* extraction with 50% AA at a dose 0.0002% on eight asthenozoospermic patients is shown in Figure 2. B. The progressive sperm motility grade (GA %) revealed no increase after activation (3.12%) with the extract compared with before activation (3.12%). The GB grade percentage of sperm motility was 40% before activation compared with 35% after activation. The normal sperm morphology percentage was increased significantly (P<0.05) after activation (55%) in comparison with 38.75% before activation.

Effect of 80% EtOH, 60min. Extraction (0.002%)

The result of eight asthenozoospermic patients is presented in Figure 2. C. It is shown through these results that the GA% significantly increased (P<0.05) after activation (15.63%) in comparison with 1.25% before activation. The GB% grade was 42.5% after activation compared with 47.63% before activation. The total of GA + GB% was 58.123% after activation, whereas that before activation was 48.88%.A highly significant increase (P<0.001) was found in the normal sperm morphology percentage from 32.5% before activation to 67.5% after activation.

Effect of 80% MeOH, 60min. Extraction (0.002%)

The effect of *Tt* extract at the mentioned dose level and solvent on twelve patient's sperm parameter is presented in Figure 2.D. The GA% was increased significantly (P<0.05) after activation (17.5%) compared with that before activation (0.83%). The linear sperm motility GB% decreased significantly (P< 0.05) after activation (26.67%) in comparison with that before activation (43.42%). The sum of GA + GB% exhibited nearly the same value before and after activation (44.24 and 44.17% respectively). The normal morphology of sperm was significantly (P< 0.05) increased from 33.33% before activation to 60.42% after activation. Comparison of the activity of the different extracts of *Tt* at different dose levels on the asthenozoospermic parameters is shown in Figure 3.

These results showed that H_2O extract for 15min. at a dose 0.02%, 50% AA extract for 60min. at a dose 0.002%, 80% MeOH extract for 60min. at a dose 0.002%, 80% EtOH extract for 60min. at a dose 0.002%, 80% MeOH extract for 15min. at a dose 0.0002% and 50% AA extract for 15min. at a dose 0.002% exhibited the highest activity, whereas, 80% MeOH extract for 15min. at a dose 0.0002% and 50% AA extract for 60min. at a dose 0.0002% showed the lowest activity.

Discussion

Many research's dealing with the extraction of different vegetal materials using the ultrasonically assisted extraction were found in the literatures. The use of low frequency sonication is very important for the herbal constituent's degradation since such frequency could be used for the release of the medicinal compounds. Such extraction procedure by the use of sonication could involve the change in permeability of the cell wall, the break of the cell wall and the release of the cell contents once the wall was

broken [14,15]. Among the benefits of the ultrasonic extraction are the hydrolysis processes which occur simultaneously with the fragmentation of the vegetal materials [24, 26]. Previous studies claimed that treatment of male mice with crude aqueous extract of Tribulus terrestris revealed a significantly increased sperm concentration and grade of motility [27]. The effect of adding *Tt* extract to the water of cocks was found to improve cocks semen quality and increased spermatozoids concentration [28]. Other authors showed revealed a significant effect of *Tt* extract on ram's spermatogenesis [29], while administration of lyophilized aqueous extract of *Tt* to albino rats revealed no significant effect on the sperm count [30].

Many active ingredients have been demonstrated in *Tt*, among which is protodiocin, a compound that enhance erection, libido, spermatogenesis, ejaculatory volume, sperm motility, muscle mass and testosterone hormone in both human and animal models [31,32]. To the best of our knowledge, very limited reports can be traced in the literature dealing with the activity of *Tt* extract on human sperm quality *invitro*. A study conducted in our laboratory using the aqueous and ethanolic crude extracts obtained via conventional extraction (in doses comparable to those used through the present study) using the same parts of the plant showed no positive activity on human sperm when activated in vitro[33].

However the present study has demonstrated that ultrasonically assisted extraction of *Tt* at a low frequency (20KHZ) using a different solvent enhanced the progressive motility, the nonlinear motility and the normal morphology of human asthenozoospermic semen samples *in vitro*. Our results showed that the aqueous extract at a dose 0.02%, 50% AA at a dose 0.002% and both EtOH and MeOH at a dose of 0.002% obviously enhanced the sperm motility and sperm morphology. In our study, it is possible that ultrasonic extraction of the aerial parts of *Tt* with the solvents used namely, H_2O, AA, MeOH and EtOH can enhance the release of the active constituents from the plant more than the conventional extraction does and then enhance its activity on sperm activation. Comparison of the extraction periods showed that both the 15 and 60 min. gave a good result with respect to the progressive sperm movement (GA%) and the improvement of the sperm morphology by using the four solvents, with the H_2O was only active after 15 min. extraction periods at a dose 0.02%, whereas the other solvents were active after the two period of extraction mainly at the dose 0.002%. Asadmobini *et al.*, 2016 [34] demonstrated that *Tt* aqueous extract, obtained by conventional extraction, has a remarkable effect on both the viability and the motility of the human sperm *invitro* at doses 40 and 50 µg/ml when incubated for a period of 60 and 120 min. Since the antioxidants have a great effect on sperm motility [35].

Tt extract contains polyphenols including a wide class of compounds such as phenolic acids and flavones [36, 37] and coumaroylguinic acid derivatives [38] which possessing a high antioxidant activity and assumed to be the main ingredients responsible for the antioxidant activity of the plant and could be responsible for the effective mechanism on human sperm parameters improvement. Furthermore, Nassar *et al.*, [39] suggested that a remarkable effect on human sperm motility might have a relation with the trace element especially Ca^{+2} which is available in *Tt*. This trace element could inhibit the enzyme phosphate diesterase, which prevent cyclic adenosine monophosphate degradation and enhance sperm motility [40].

Conclusions

The current investigation confirmed that low use of low frequency assisted ultrasonic extraction may be superior on the conventional extraction in releasing more active ingredients of the vegetal materials and then exhibiting a good enhancement in the different sperm parameters including sperm motility and sperm morphology which are very important factors in assisted reproductive techniques. The antioxidant activity of the plant and the trace element content especially Ca^{+2} could be responsible for the effective mechanism on human sperm parameters improvement. It is also concluded that the aqueous extract was active after 15 min. extraction period at a tenfold higher dose than other solvents, whereas the AA, EtOH and MeOH solvents were active after both extraction periods at low doses. Further studies are required to investigate the sperm quality; fertilization capacity and embryonic development after exposure to *Tt* extract *in vitro*.

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