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PHYTO-PHARMACOLOGICAL STUDIES ON ETHANOLIC EXTRACT OF PUERARIA TUBEROSA AND ITS EFFECT ON SPERMATOZOA PARAMETERS.

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Abstract:
It is widely acknowledged that over 80% population of developing countries cannot afford the allopathic medicine. Around the globe, medicinal plants are used as folk medicines to heal variety of disorders. The medicinal plants possessing aphrodisiac properties are extensively used for the management of reproductive disorders and have significant advantages over conventional allopathic prescriptions. In current study, *Pueraria tuberosa* was evaluated for Phytochemical present and therapeutic uses like antioxidants and aphrodisiac potential. Ethanolic extract of tubers was prepared and proceeded for qualitative analysis the results shows considerable amounts of phytomolecule as flavonoids, tannins, phenols, alkaloids and minerals as Fe, Mg, Cu, Zn etc. are present. Moreover quantitative phytochemical analysis through standard protocols was also performed and the finding of TPC, TFC and TSP are 24.27 ± 0.348, 10.41 ± 0.032 and 2.234 ± 0.006 respectively. The antioxidant potential through DPPH, FRAP, ABTS and the findings are 49.79, 22.48 and 33.27, the antioxidant enzymes were also evaluated and the results are satisfactory. The heamolytic assay shows *P. tuberosa* was safe upto 1000mg concentration as it produced minimal cell lysis. It was found that incubation of the semen with different concentrations of Ethanolic extract of *P. tuberosa* enhanced the motility and preserved the viability of spermatozoa in dose dependant manner. This study may open new horizon for researchers to explore it further and clinical practitioners to treat oxidative stress-related diseases as well as infertility by this natural drug.

Key Words: *P. tuberosa*, Phytochemical, Antioxidants, Reactive Oxygen Species, Spermatozoa.

Introduction:

It is widely acknowledged that over 80% population of developing countries cannot afford the allopathic medicine. According to WHO, they rely on conventional herbal remedies to cure a variety of ailments (Zhang & Organization, 2002). Due to the therapeutic potential of medicinal plants in treating a variety of medical issues, demand for beneficial herbal sources has grown steadily worldwide (Choudhury et al., 2018). Herbal medicines compare to allopathic medication have fewer adverse effects. Pakistan have a rich repository of plant life due to its diverge and favorable environment (Gilani, 2005). Approximately 6000 plant species are documented in Pakistan, including 600 to 700 medicinal species(Ullah, 2017).

Ancient communities depend on ancestral wisdom and traditional knowledge passed down through generations for the optimum utilization of local flora.(P. Gupta, Sharma, & Sharma, 2014).Medicinal plants are abundantly found in Asian countries have been used for thousands of years and still are utilized in contemporary time. The utilization of medicinal plants as folk medicines is also privileged in Pakistan to heal variety of disorders particularly chronic cases (Kensa & Yasmin, 2011; Taid, Rajkhowa, & Kalita, 2014). The medicinal plants possessing aphrodisiac properties are extensively used for the management of reproductive disorders and have significant advantages over conventional allopathic prescriptions (R. Singh, Ali, Gupta, Semwal, & Jeyabalan, 2013). These herbal medicines are renowned for their harmless effects and efficient healthcare characteristics. The aphrodisiac agents are believed to boost sexual activity by stimulating specific levels of neurotransmitters or certain sex hormones like testosterone which change physiology of the body (Malviya, Jain, Gupta, & Vyas, 2011). (Patel, Kumar, Prasad, & Hemalatha, 2011; Semwal, Kumar, & Singh, 2013; A. P. Singh, Sarkar, Tripathi, & Rajender, 2013). *P. tuberosa* belong to Fabaceae family widely used in traditional medicine as an aphrodisiac plant. *P. tuberosa* is a perennial plant and is widely found in Pakistan and Nepal(Egan, 2020). Traditionally, the leaves and tubers of this plant are believed to have medical properties. According to previous literature, the tuber is utilized in multiple herbal preparations as a restorative tonic, spermatogenic, antiaging, and immune booster(Pandey, Srivastava, Dwivedi, Upadhay, & Singh, 2019). It has also been proven to be effective in hepato-splenomegaly, sexual debility, menopausal syndrome, spermatorrhea, and fertility disorders(Kanthaliya, Joshi, Meena, & Arora, 2021). The plant has several therapeutic effects that include antioxidant, anti-inflammatory, antiulcerogenic, anticonvulsant, antidiabetic, cardioprotective, nephroprotective, nootropic, neuroprotective, and restorative properties(Maji, Pandit, Banerji, & Banerjee, 2014).

The *P*. tuberosa tubercontains numerous isoflavonoidsbioactive constituents like genistein, puerarin, tuberosin, and daidzein. Various other pharmacological constituents are mangiferin, isoorientin, biochanin A, biochanin B, and irisolidone(Bharti, Chopra, Raut, & Khatri, 2021). The broad spectrum phytochemicals advocate further studies to discover therapeutic potential of this plant. The methanol components of the plant are thought to be responsible for the suppression of lipid peroxidation as well as superoxide scavenging and hydroxyl radical activities(Lencina, De Simone, & Cunico Filho).Furthermore, daidzein and puerarin mimicked a-tocopherol in their antioxidant activities(Epriliati & Ginjom, 2012). The extracts of the tuber were also observed to have therapeutic effects against oxidative damage due to the pharmacological actions of daidzein and genistein (Maji et al., 2014). These constituents are also deemed to prohibit low-density lipid oxidation. Furthermore, a flavonoid compound which is named tuberosin has been isolated in the near past and it is believed to have rigorous scavenging activities against several free radicals.

P. tuberosa also has a positive effect on the sexual behavior of male rats. The tuber is used for aphrodisiac, rejuvenating properties, and longevity(Maji et al., 2014).*P. tuberose* is commonly prescribing to treat sexual weakness and inflammation. It has also several other therapeutic activities, including cardiotonic, diuretics, and galactagogue agent (Egan, 2020). It is widely used in herbal formulations such as health tonic. In context of previous literature and increase prevalence of male sexual problems, this plant was selected to perform this study. The aim of this study was to develop

plants based alternative medicines with lesser side-effects, economically cheap, easily available and with excellent compatibility. In this context present study was planned to find out potential remedy having beneficial effects on spermatozoal parameters and fertility enhancing effects.

Material and Methods:

Collection, Authenticity, Processing and Extraction of Plant Material

P. tuberosa roots were purchased from the local market of Faisalabad and authenticated from the Department of Botany, Govt. College University Faisalabad under voucher No. GBM-236/21.The roots were examined for contamination and adulteration. Then mechanically crushed into coarse powder(Likhitkar & Pande, 2017).The grind roots were extracted with ethanol solvents by microwave assisted extraction (MAE) process. 100g of the drug was soaked in a 500ml glass beaker for a day. Then heated in a microwave oven for 6 minutes at low power and kept to cool with stirring. The process was repeated 5 times and filtered through the Whatman filter paper. Ethanol was evaporated in rotary evaporator under reduced pressure. The thickened extract was further evaporated at 55C$^{\circ}$ in a water bath to a get dry extract which was stored in a refrigerator for further use in experiments.

Phytochemical Evaluation

For phytochemical analysis stock solution was prepared by dissolving half gram of ethanolic*P.tuberosa* extract in twenty mL of ethyl alcohol.

Qualitative Evaluation of Phytochemicals.

Qualitative phytochemicals analysis of *P. tuberosa* was performed for tannins, saponins, flavonoids, steroids, alkaloids and glycosides following the standard protocols cited by(Fischer, Isman, & Stafford, 2012).

Quantitative Phytochemical Evaluation of *P. tuberosa*.

Determination of Total PhenolicContents (TPC)

The Folin–Ciocalteu reagent assay was used to determine the total phenolic contents. Samples (100µL) extract was mixed with 0.5ml folin–Ciocalteu reagent previously diluted with 7ml of deionized water. The solution was allowed to stand for 3min at 25ºC, and 0.2ml of a saturated sodium carbonate solution was added. The mixture was allowed to stand for another 120 minutes in a dark place for the completion of the reaction and formation of complexes. The absorbance of all aliquots was measured at 725 nm. Gallic acid was used as a standard for the calibration curve. The total phenolic content of the extract was calculated in terms of gallic acid equivalent (GAE) per gram of dry extract using the standard curve equation. Total phenolic content was expressed as mg Gallic acid equivalents per gram of dry extract of plant samples.

Determination of total flavonoid contents (TFC)

The total flavonoid content was determined by the aluminum chloride colorimetric method. The content of flavonoids was determined as quercetin equivalent. 1 ml of plant extract in the respective solvent (stock solution SS) was mixed with 2 ml AlCl3 (2% w/v) in methanol and the solution was made up to 25ml with a methanolic solution of acetic acid (0.5% v/v) (Probe solution PS). 1ml of SS was made up to 25ml with a methanolic solution of acetic acid (contrast solution CS). The absorbance of PS and CS was measured at 420nm after a 30-minute. The result was expressed as mg Quercetin Equivalent per g dry extract.

Determination of Total Soluble Proteins (TSP)

Following Bradford's (Hameed et al., 2021) instructions, total protein content in 0.1 g of fresh *Pueraria tuberosa* tubers was determined by homogenizing the sample in 2mL of phosphate buffer saline (pH 7.2) and centrifuging it for 10 minutes (MIKRO-200 R; Hettich GmbH and Co. KG) for thirty minutes, the sample was kept at room temperature for incubation. A UV-VIS spectrophotometer

(Hitachi U-2910, Tokyo, Japan) was used to estimate the mixture's optical density (OD) at 595 nm, and BSA (Bovine serum albumin) served as the standard.

Fourier-transform infrared (FTIR) Spectroscopy

The presence of various kinds of chemical bonds or functional groups in the phytochemicals was determined by using a Fourier Transform Infrared Spectrophotometer (FTIR). The distinguishing characteristic of chemical bonding is the wavelength of light that they absorb and the chemical bond can be evaluated on interpreting the infrared absorption spectrum. For FTIR analysis etahnolic plant extract in dry form were used. To prepare the pellets of extract for analysis, potassium bromide (KBr) powder was used to mill with dried extract powder (10 mg) and pressed to make pellets. After making the pellets The Central Hi-Tech Laboratory, Government College University, Faisalabad, Pakistan, has an FTIR spectrometer in the frequency range of 400-4,000/cm that was used to identify functional groups as representative of a wide variety of significant phytochemical constituents (Model Bruker Platinum ATR with accessories A225/Q Platinum ATR Multiple Crystals CRY diamond and having Interferogram size of 10550 points).

Trace Elements (Heavy metals) estimation

Significant trace elements concentration in the examined medicinal plant was determined using an atomic absorption spectrophotometer located in the Central Hi-Tech Laboratory of the Government College University in Faisalabad, Pakistan. The process used by the AOAC (2000) was divided into two steps, with nitric-perchloric acid being employed to digest the material in the first phase. In the 1st phase, 250 mL capacity beaker was taken and one gram of wet sample and concentrated HNO_3 (10 mL) were taken. Hot plate was used to boil the mixture to oxidize all easily oxidizable materials for 30-45 min.

Then mixture was left to cool and then adding 5 ml of 70% $HClO_4$, the liquid was heated once more until thick white vapors emerged. This mixture was cooled and then after adding 20 mL of distilled water, the liquid was heated once more to expel any odors. After cooling the solution, Whatman No. 42 filter paper and <0.45 lm Millipore filter paper were used to prepare the filtrate in a 50mL volumetric flask and filtrate was diluted to 50mL with distilled water. In 2nd phase, after digestion (Colagar, Marzony, & Chaichi, 2009)method was utilized to determined heavy metals (ppm) including Fe, Mg, Cu, Pb, Cd, Mn, Ni, Co, and Zn by an atomic absorption spectrometer (AAS) (Aurora, Canada).

Evaluation of Anti-Oxidant Activity

DPPH radical scavenging activity

Free radical scavenging effect was determined by using the free radical generator DPPH (2, 2-diphenyl-1-picrylhydrazyl) by a similar method to Brand-Williams, Cuvelier, &Berset, 1956. 0.1mg solution of DPPH in methanol was prepared and 1ml of this solution was added to different concentrations (6.25, 12.5, 25, 50, 100 & 200µg/mL) of plant extract to 3 ml of the solution of all extracts in methanol. Standard DPPH solution was then mixed with test extract with dilution in a ratio of 1:3. The mixtures were kept in the dark at room temperature for 90 min and the absorbance was measured at 517 nm using UV-VIS spectrophotometer (Genesys 10S UV Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of the reaction mixture indicated higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated by using standard formula and each test was carried out in triplicate, the outcomes were averaged.

Antioxidant activity by radical cation (ABTS)

The ABTS assay was performed using a slightly modified version of the method that Huang described (Huang et al., 2021). When 7.45 mM potassium persulfate was mixed with the ABTS solution, ABTS+ cations were formed. The mixture was diluted with ethanol until it had an absorbance of

0.70+0.02 at 734 nm prior to use. After being in the dark for 90 to 120 min at room temperature the Genesys 10S UV-VIS (Thermo Scientific) was used to measure absorbance at 734 nm precisely six minutes after adding 100 mL of the sample or the Trolox standard to 3.9 mL of diluted ABTS+solution. The IC50 was calculated using the standard curve equation of sample concentration and RSA percentage, and the results were expressed as (TEAC) Trolox equivalent antioxidant capacity. The calculations were made by using standard formula and results were averaged after the entire test performed in triplicate.

Ferric reducing activity power of plant extract (FRAP).

The power of reducing the extract was determined using the method developed by (Müller, Fröhlich, & Böhm, 2011). One mL of solution containing 10, 20, 30 & 40 µg/mL of extract was mixed with phosphate buffer (2.5ml, 2M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated for twenty minutes at 50ºC. Moreover 2.5ml of trichloroacetic acid (TCA, 10%) was added to the mixture and centrifuged at 1500 rpm for 10 min. Upper layer of solution (2.5 ml) was mixed with FeCl3 (0.5 ml, 0.1 percent) and distilled water (2.5 ml) and the absorbance was estimated to be 700 nm. The increased reducing power was indicated by the reaction mixture's increased absorbance. The result was presented as RSA %age while IC50 was calculated by using the standard curve equation of sample concentration and RSA %age. The calculations were made by using the standard formula and results were averaged after taking readings in triplicate.

Antioxidant enzymes assay.

Fresh *Pueraria tuberosa* tubers were grinded in a mortar and pestle in the presence of cooled phosphate buffer (50 mM;) for the antioxidant enzyme extraction. dithiothreitol (1 mM) and pH-7.0. The supernatant from the centrifugation of this solution at 25200 rpm for approximately 20 minutes at $4C^{o}$ was used to measure the antioxidant and enzyme activities following method coated by (Galal, Raman, & A Khan, 2015).

Superoxide dismutase (SOD)

With minor modifications SOD activity for *Pueraria tuberosa* roots was determined by method developed by Gong et al. as coated by (Chang et al., 2022) .For 15 minutes at 78 µmol m/2/s, the glass vials containing the reaction mixture were illuminated with 15 watts of fluorescent light and at 560 nm, the absorbance was measured.

Catalase (CAT), peroxidase (POD) contents

In accordance with Cakmakk et al.'s method (Abbasvand, Hassannejad, Zehtab-Salmasi, & Alizadeh-Salteh, 2020), by min or modifications the CAT and POD activities were performed. In every 20 second the absorbance of the reaction mixture was estimated at 240 nm. Following the breakdown of H_2O_2, the absorbance's of the CAT and POD reaction solutions decreased at 420 and 470 nm, respectively. Units of enzyme activity were expressed as mg-1 of protein (U = 1 mM of H_2O_2 reduction min-1 mg-1 protein).

Hemolytic assay to determine cytotoxic potential of *Pueraria tuberosa* extract

The (Powell, Catranis, & Maynard, 2000) technique was modified to employ the hemolytic test to assess the safety status of ethanolic plant extract. 10mg of studied plant extract was dissolved in 1mL of DMSO (20%) to check the cytotoxicity. Fresh blood from healthy volunteer was collected in heparin added falcon tube and centrifuged to remove the acellular part of blood for 5 min at 3000 rpm. After removing the supernatant, phosphate buffer saline (pH 7.4) was used to wash the RBCs three times at 4ºC and RBCs suspension was prepared with 7.068 x 10^8 RBCs/ mL (using heamacytometer) by PBS before assay. Then 180 µL of RBCs suspension was taken in eppendrof tubes (2mL) labeled respectively and 20 µL of respective plant extract was added (20 µL). After mixing slightly mixture was incubated for 40 min at 37ºC and agitated repeatedly then cooled after incubation using ice cubes for 5 min. Then all the tubes were centrifuged for 5 min at 3500 rpm to

obtain the supernatant. After that, supernatant (100 µL) was diluted with cooled PBS (900 µL), and 200 µL of combination was gently mixed before being put onto 96-well plates with labels to measure the absorbance of RBCs treated with triton X-100 (0.1%) as the positive control and PBS as the negative control. Three duplicates of each combination were read using a Micro Well Plate Reader Bio TeK, Quantset at 576 nm wavelength. The percentage of red blood cells that were hemolyzed by the plant extract was calculated using the method below:

Percent Hemolysis = $\left[\frac{Ae - Ap}{Ad\text{-}Ap}\right] X\ 100$

Here: Ae= plant extract absorbance; Ap= PBS absorbance; Ad= DMSO absorbance (20%) used to make plants dilution (Powell et al., 2000).

***In vitro* spermatozoa parameters**

Healthy participants were picked for the collection of semen samples after receiving written permission, and samples were studied in accordance with World Health Organization (WHO) procedure for the measurement of in vitro spermatozoa characteristics (WHO, 2010). On the evaluation of semen quality only the samples used for the current analysis were normal. According to WHO semen from human having 2 mL volume or more/ ejaculate with pH 7.2–8.0, spermatozoa count as 20×10^6 spermatozoa/mL or higher, per ejaculate 40×10^6 spermatozoa or high, with motile spermatozoa 50% or more upto 60 min after ejaculation (WHO, 2010). To examine the effects of certain medicinal compounds, crude aqueous ethanolic extracts (25 µg/mL of physiological saline) were utilized to dissolve the compounds in labelled Eppendorf tubes, which were then left at room temperature for an entire night.

To ascertain the in vitro impact of *Pueraria tuberosa* extract on one or more semen parameters, such as viability (%), motility total (%),and progressive motility (%), (Sander & Cramer, 1941) procedure was modified. An identical volume (1:1) of semen was combined with plant extract diluted in 0.9% physiological saline after semen analysis was collected and evaluated for normal criteria. The sperm motility total (%) and progressive motility (%) were then analysed under a light microscope (40X) at predetermined intervals, including at 0min, 15min, 30min, 45min, 60min, and 120min. Using 0.1% Eosin Y stain in 0.9% physiological saline, the effect of the researched medicinal herbs on spermatozoa viability (%) was also tested. The semen, extract, and stain were incubated in a 1:1:1 ratio for up to 120 minutes as described above (Cheesbrough, 2005), (Prakash, Ravikumar, Reddy, & Kannapiran, 2014).

Statistical analysis

Obtained results were presented in the form of Means ± Standard Deviation (SD) graphically and tables using Microsoft ® office and Excel Version

Results

Phytochemical Analysis

Qualitative Phytochemical Analysis

Phytochemical analyses of the ethanolic extracts of *P. tuberosa* contained flavonoids, alkaloids, glycosides, tannins in major concentrations while saponins, steroids, and triterpenoids were in less concentration as shown in table 1.

Table 1 Qualitative phytochemical screening of ethanolic extract of *P. tuberosa*.

Sr #	Phytochemical	*P. tuberose*	Test Name
1	Flavonoids	+++++	Shinoda's test
2	Glycosides	+++	Molisch's test
3	Steroids	+++	Liebermann-burchardt test
4	Tannins	+++++	$FeCl_3$ test
5	Saponins	+++	Frothing test
6	Alkaloids	++++	Dragendorff's test
7	Anthraquinones	++	Borntrager's test

+++++ = Maximum, ++ = Minimum

Quantitative Phytochemical Analysis of Plants extracts

Total phenolic and flavonoids contents

The total flavonoid and phenolic contents of *P. tuberose* ethanolic extract are given in Fig. 1-A and table 2. Significant ($p<0.05$) statistical difference between TPC was observed in the ethanolic extracts of studied medicinal plants. The presence of significant amount of TFC in the studied medicinal plant revealed new natural sources to compete the disorders may cause due to imbalance antioxidant status.The standard curve was drawn with the concentration and absorbance of gallicacid to calculate the total phenolic contents of *P. tuberosa*as gallic acid equivalent. This was found as 24.27 ± 0.348 in case of *P. tuberosa* dry extract of the sample.

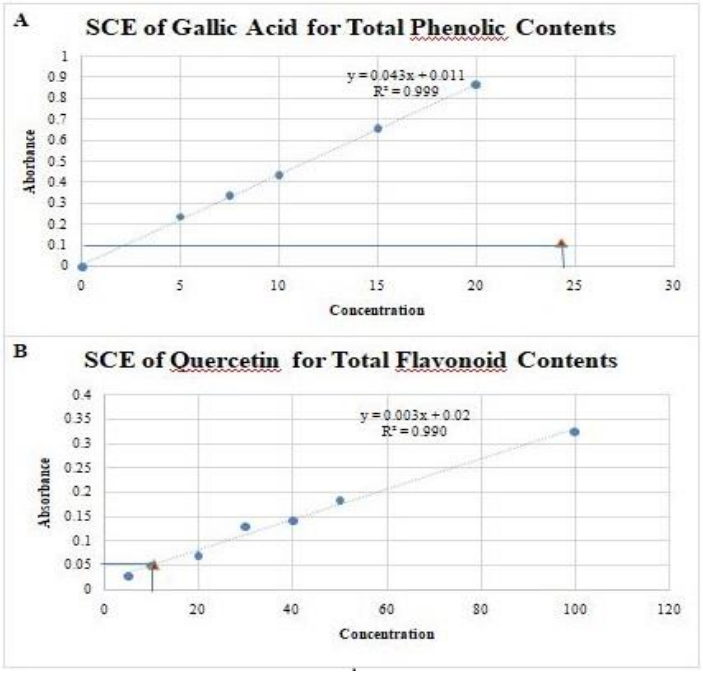

Figure-1 (**1-A.** Standard curve of Gallic acid for TPC and dot represents the phenolic contents found in ethanolic extract of *P. tuberosa).* While (**1-B.** Standard curve of quercetin for flavonoids represents the flavonoid contents found in ethanolic exttact of *P. tuberosa).*

The standard curve equation in figure 1.B was used to convert the *P. tuberosa* tubers extract's total flavonoids (TF) into quercetin equivalent, and Table 2 lists the TF value 10.41 ± 0.032 in dry extract of study pants part used.

Total Soluble Proteins

The total soluble proteins (TSPs) level in tubers extract of *P. tuberosa* as 2.234 ± 0.006 which are shown in Table 2.

Table-2. Phytochemical Constituents and antioxidant activities of *P. tuberosa* ethanolic extract as mean ± SD of multiple determinations of each experiment.

Plants\Contents	***P. tuberose***
TPC (mg GAE/g dry plants material)	24.27 ± 0.348
TFC (µg QE/g dry plants material)	10.41 ± 0.032
Total Soluble Proteins (mg/g dry plant material)	2.234 ± 0.006

Fourier-transform infrared spectroscopy (FTIR)

Fourier-transform Infrared spectroscopy is commonly used to explore various functional groups in the chemical constituents might be responsible for their pharmacological activities. Table 3 represent the linkage between possible function group and specific wavelength a molecule absorbed. The absorption peaks at range 4,000 to 2,500/cm was associated with the stretching vibration of single bond formed by hydrogen and other element like O-H, N-H and C-H; peaks at 2500 to 2000/cm explored the absorption of light due to triple bond e.g. C≡C, C≡N; peaks at 2000 to 1500/cm represent the presence of compound with double bonds including C=C, C=O while absorption peaks at 1500 to 400 /cm consisted of many complicated bands and this part of the spectrum is unique to each compound also called fingerprint region. It is rarely used to identify fuctional groups like C-C, C-N, C-O, C-Cl, C-I, S-S and N=O*.

Figure-2 of selected medicinal plant extracts' FTIR analyses revealed absorption peaks at 2,500–4,000/cm, which indicated the presence of both intra- and intermolecular hydrogen bonding. The asymmetrically stretched vibration of C-H was thought to be responsible for the absorption peak at 2,918/cm. Perhaps the stretching vibration of C-H is what causes the peak at 2924 cm, 2853 and 2922 cm. The symmetrically stretched vibration of C=O was identified as the source of the absorption peak at 1619/cm and 1604/cm. The stretching vibration of C=N and C=C may be connected to the absorption peaks at 1,518/cm. Strong to medium vibration of the C=C and N=O functional groups may be responsible for the peaks at 1400 to 1500/cm (1,448/cm and 1,442/cm). The stretching vibration of C-O was responsible for the absorption maxima at 1,228/cm. The asymmetrically stretched vibration of C-O was thought to be responsible for the peaks at 1089/cm, 1079/cm, 1075/cm, 1056/cm, 1015/cm and 995/cm . While the peak below it may be connected to vibrational S-S stretching, the absorbance peak at 764/cm may indicate aliphatic C-I stretching as shown in (Table 3, Fig.-2).

The findings of our research represented that selected medicinal plants contained wide range of function groups which ultimately represented the presence of various active phytocompounds in the selected herbs. The major purpose of FTIR was to explore the active functional groups and it was also first time conducted on selected medicinal plants.

Table-3 Different functional groups and possible phytochemical constituents identified using FTIR in ethanolic extract of *Pueraria tuberosa*.

Wave number (cm^{-1})/ Possible functional groups	***Pueraria tuberosa***
4,000 to 2,500/cm O-H, N-H and C-H Stretch vibration	3268.9
	2922.2
2000 to 1500/cm C=C, C=O, C=N Stretch vibration	1619.5
1500 to 400 /cm C-C, C-N, C-O, C-Cl, C-I, S-S and N=O* Stretch, Bend or scissoring, rock vibration	1148
	1075.3
	995.2

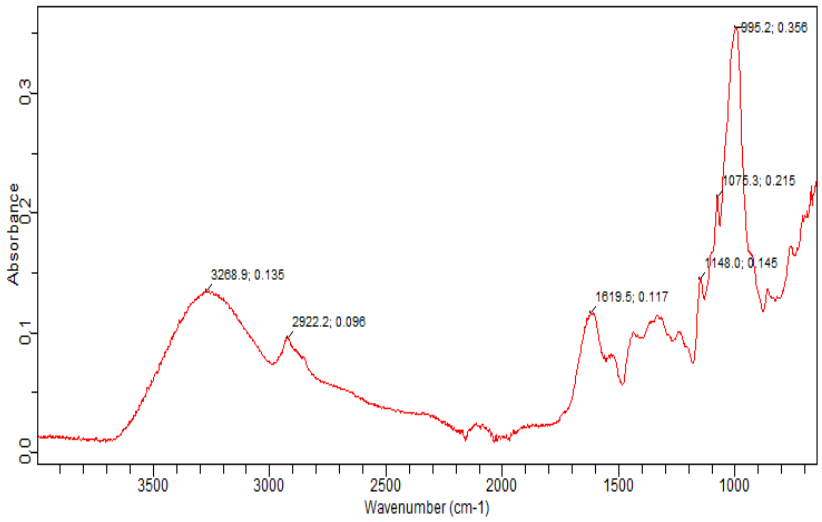

Figure-2 FTIR spectrophotograph representing possible functional groups and potential phytochemical components of the ethanolic extract of *Pueraria tuberosa* are shown.

Mineral contents of *Pueraria tuberosa*

In the current research work mineral contents both essential and toxic elements including Cu, Fe, Cd, Pb, Zn, Ni and Mn etc. were investigated in selected medicinal plant. The results revealed that medicinal plant contained significant ($p<0.05$) concentration (ppm) of minerals as given in Table-4. it reveals that *P. tuberosa* contained mineral from high concentration to low concentration in following order Iron (Fe), Manganese (Mn), Copper (Cu), Nickle (Ni), Lead (Pb), Zinc (Zn), Cadmium (Cd). These results shows heavy mentals present in this extract in only very minute quantity as of Pb. etc.’’’’

Bioelements are classified in different groups as Group I to Group V based on their requirements and applications(Munir et al., 2022). It was found that mineral perform a wide range of vital role in our body like hemoglobin structure required iron, similarly various antioxidant enzymes required Fe, Mn etc. as prosthetic group for their activities. Similarly, Zn is also required for healthy antioxidant agent both in male and female body to protect spermatocytes and oocytes from free radicals and ROS (Fusco et al., 2021). Moreover, evaluation of natural medicines for toxic minerals like Pb and Cd is very significant before putting them in clinical trials.

Table-3 Mineral contents of *P. tuberosa* tubers Ethanolic extract as mean ± SD of multiple determinations of each experiment.

Plants\ Contents	***P. tuberosa***
Copper (ppm)	5.69 ± 0.113
Iron (ppm)	218.17 ± 5.37
Zinc (ppm)	0.5457 ± .013
Manganese (ppm)	18.27 ± 0.41
Nickle (ppm)	3.18 ± .32
Cadmium (ppm)	.0126 ± 00
Lead (ppm)	0.76 ± .03

Antioxidant activities of *Pueraria tuberosa* extracts

The antioxidant phytochemical components can stop the oxidation of substrates and aid in the detoxification of free radicals like reactive oxygen species (ROS), which may be created in our bodies because of oxidation processes. Due to an inadequate antioxidant system, the human body becomes unable to control oxidants, which causes oxidative stress and several metabolic and other health issues (Smolskaitė, Venskutonis, & Talou, 2015). Currently, a wide range of herbal antioxidants including

phenolics, carotenoids, flavonoids, vitamins, and food supplements are used to improve the antioxidant status in the body which significantly compete the oxidative stress in human body (Jayakumar & Muralidharan, 2011). The selected medicinal plants were evaluated for their antioxidant potential by using following parameters which explored the significant ($p<0.05$) antioxidant activities of selected medicinal plants.

Total antioxidant capacity (TAC) by DPPH scavenging and ABTS scavenging methods

The antioxidant capacity of phytochemicals is not only due to the donation of electrons to hydrogen but also could generate intermediate stable molecules. It was well reported that herbal preparations have potential antioxidant activities particularly due to phenolic compounds ((Cai, Luo, Sun, & Corke, 2004); (Loo, Jain, & Darah, 2008)). During current research work for the measurement of medicinal plant potential to scavenge the free radicals' different parameters includes TAC by DPPH and ABTS scavenging assays were used and the results of DPPH and ABTS are given in Table-5.

DPPH radical scavenging assay

DPPH is frequently used to measure how well *Pueraria tuberosa* neutralizes free radicals when assessing a substance's ability to do so. DPPH was utilized as a free radical-generating reagent. At 517 nm, antioxidants from *Pueraria tuberosa* were able to transform DPPH into yellow diphenyl-picryl hydrazine. As the outcomes, the IC50 value and RSA percentage have been calculated. Table-3 compares the results of the DPPH radical scavenging test with the Ethanolic extract of under study plant and standard (M. Gupta, Sasmal, Karmakar, Sasmal, & Chowdhury, 2016).

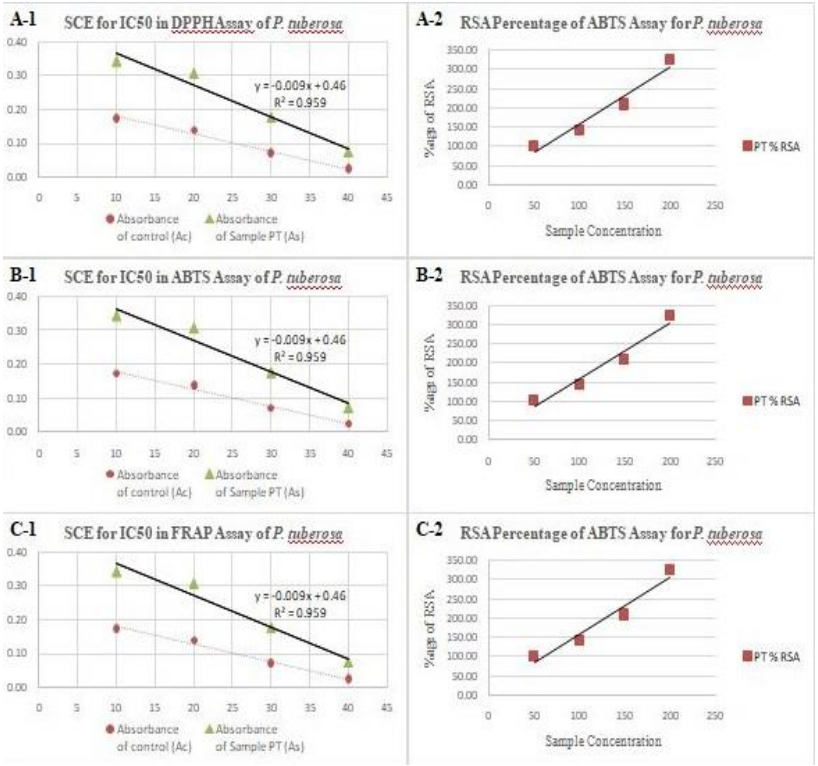

Figure-3 **A1** Standard curve of standard/control and *P. tuberosa* for DPPH assay. **A2** RSA percentage of study plant for DPPH assay. **B1** Standard curve of standard/control and *P. tuberosa* for ABTS assay. **B2** RSA percentage of ABTS assay for *Pueraria tuberosa,* **C1** Standard curve of standard/control and *P. tuberosa* for FRAP assay, **C2** Radical scavenging activity by *Pueraria tuberosa*.

Ascorbic acid served as a control so that the standard curve could be plotted as it is determined in Figure 3 The IC50 of *Pueraria tuberosa* was 49.79 while RSA was found 90.09± .37 (mg/ml) at 50g concentration as represented in Table 5 and Fig. 3 A2.

ABTS radical scavenging assay (TEAC assay)

Sodium persulphate converts ABTS to cationic radical bluish in color and absorbance was taken at 734nm. Most of the antioxidants are reactive to the ABTS radical cation. The bluish ABTS cation radical is converted to back during this reaction. The (TEAC) Trolox equivalent antioxidant capacity assay is the name of this test. The ABTS extremist rummaging consequences of the ethanolic concentrate of *pueraria tuberosa* in correlation with the normal (Trolox) have been determined in Figure-3.

The standard curve was plotted using the inhibition percentage against various strengths of the research drug's ethanolic extract and Trolox as the standard. This curve (g/ml) showed the ethanolic extract of *Pueraria tuberosa* shows IC50 22.48 while RSA percentage was 103.16±1.02 as shown in Table 3 and Figure-3 B2. It also showed that the inhibition percentage increased with the concentration of the research plant extract in the assay mixture.

The potential of ethanolic extract of *Pueraria tuberosa* to scavenge DPPH (%) and ABTS (%) explored that these herbs have significant ($p<0.05$) potential to neutralize the impact of free radicals. The DPPH and ABTS scavenging activities produced by *P. tuberosa* tubers extract were shown in given Table-5.

Reducing potential by Ferric Reducing antioxidant Power (FRAP) Assay

Different assays are used to evaluate the capacity of natural herbs to reduce the oxygen by transferring the electron from intermediate substances and preventing the production of free radicals ultimately reducing the oxidative stress (Lü, Lin, Yao, & Chen, 2010). This reducing property is due to the presence of secondary metabolites particularly the phenolic compounds to reduce the ferric (Fe^{+3}) ion to ferrous (Fe^{+2}) form by electrons donation (Sowndhararajan & Kang, 2013). A wide range of antioxidant methods are used to estimate the antioxidant capacity of medicinal plants and ferric reducing antioxidant power (FRAP) was used for selected medicinal plants. According to this assay reduction of Fe^{3+} ferricyanide complex was measured at 700 nm and the intensity of navy-blue color produced directed related to the antioxidant activities of selected herbs (Prasad et al., 2009)). Table-5 and Figure 3 displays the comparison of the FRAP test results for the understudy ethanolic extract to the standard (ascorbic acid) at 700 nm.

The standard curve for calculating the IC50 (g/ml) of each sample was determined using the %age of inhibition against each concentration of the *Pueraria tuberosa* extract. Additionally, it was found that as the concentration of the study plant extract in the test mixture increased, so did the inhibition rate. The IC50 of the alcoholic extract of *Pueraria tuberosa* was 33.27 ± 0.39 as represented in Table 5 and Figure 3 C1.

The IC50 values as well as the standard deviation obtained for the *Pueraria tuberosa* extracts using the different techniques are discussed above are presented in Table 5. It was also found that *P. tuberosa* tubers ethanolic extract have the maximum reducing potential. Moreover, *P. tuberosa* highest antioxidant activity also is the indicative of the presence of phytochemical compounds having significantly high antioxidant capacity ((Zhao, Zhang, & Yang, 2014; Zhou et al., 2009)).

Table-3 RSA %age and IC50 values of *Pueraria tuberosa* in different assays

RSA %age and IC50 value of in different assays					
		Conc.	RSA %age	SD	IC50
Pueraria tuberosa	DPPH	50	90.09	± 0.56	49.79
	ABTS	50	103.16	± 0.17	22.48
	FRAP	40	204.17	± 0.37	33.27

Antioxidant enzymes (CAT, POD, SOD)

The CAT contents were found in the tubers extract of *Pueraria tuberosa* respectively as presented; The POD is used as a usual skin caring component in cosmetic products to remove the H_2O_2 from the tissues. The POD Enzymatic antioxidant was found 4.75 with SEM ± 0.03 units/mg in extract-1 while 5.03 with SEM ± 0.07 units/mg which is the highest as found in the other drugs used for fertility.SOD contents are also known as the antioxidant defense in the body. The SOD concentration was found 26.09 with SEM ± 0.039 units/mg by *Pueraria tuberosa* extract as shown in Figure 4.

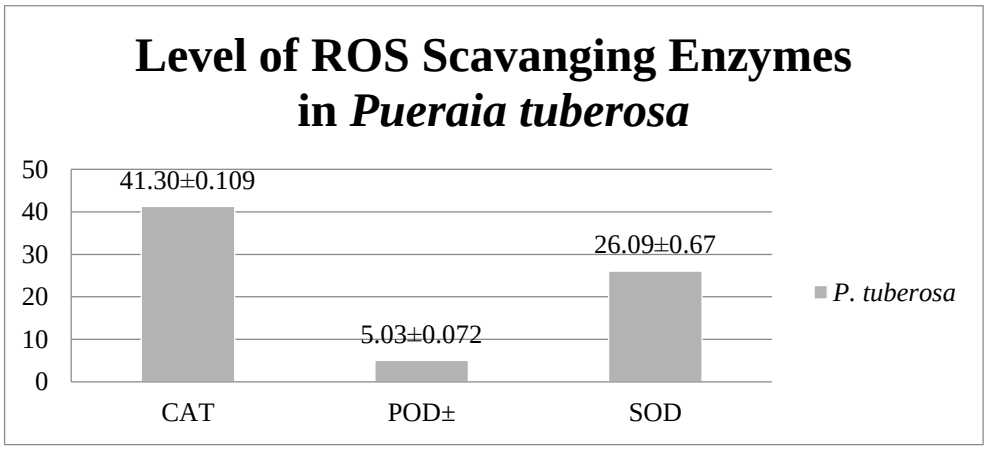

Figure 4 Levels of Reactive oxygen species scavenging enzymes in *Pueraria tuberosa*.

Cytotoxic activity of selected medicinal plant by hemolytic assay

The evaluation of safety status of a medicine in the drug development is one of the major concerns. To evaluate the toxicity of selected medicinal plants before starting the animal trial hemolytic assay using human RBCs was used (Fowles, Mootoo, Ramsewak, & Khan, 2012). RBCs are used to determine that natural herbs might have membrane stabilizing potential. *In vitro* analysis was done on RBCs from healthy human volunteers during the present project and results are represented as % age (percentage) hemolysis and expressed as Mean ± SD of triplicate measurements. Results revealed that ethanolic extract of *P. tuberosa* has minimum hemolysis (7.53 ± 1.284%) on incubating with RBCs. Positive control (Triton X-100) showed 87.43 ± 6.095% hemolysis and on the other hand negative control phosphate buffer saline (PBS) causes 3.59 ± 0.76% RBCs hemolysis as represented in Table 6 and Figure-5. Statistical analysis of the results explored that hemolysis of RBCs by medicinal plants extract is significantly ($p<0.001$) lower than the hemolysis caused by triton X-100 (positive control).

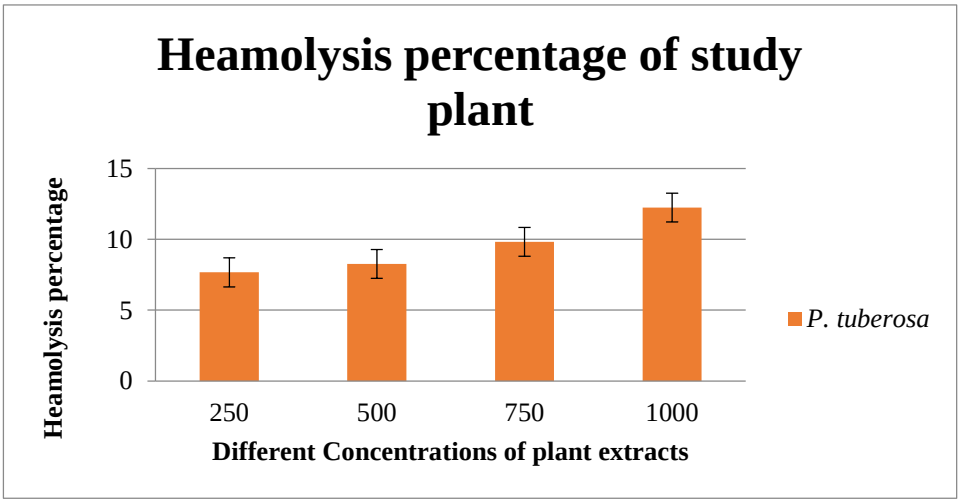

Figure-5 Hemolytic activities (%) of *Pueraria tuberosa* evaluated against washed RBCs**.**

The results are means ± SE of mean values of control and plants extract. Alphabets on the bars

represent significant ($p<0.05$) differences in group means among tested plants extract and controls.

Table-4 Thrombolytic and Hemolytic activities of the ethanolic extract of *P. tuberosa* ethanolic plant extract and controls.

Plants\Contents		**Hemolytic activity (% age)**
Puerariatuberosa	250	7.67± 0.82
	500	8.26± 1.01
	750	9.82±1.28
	1000	12.24 ± 2.105
Triton X-100		87.13 ± 6.946^{A}
PBS		4.54 ± 0.756^{D}

Means with standard deviation in the same column indicate significant ($p<0.05$) differences among tested plant extract.

***In vitro* spermatozoa parameters**

As selected medicinal plants have significant antioxidant properties and have various biologically active ingredients so their ethanolic extracts were tested for *In vitro* spermatozoa parameters. Moreover, one of the major objectives of this research work was to explore the aphrodisiac potential using *in vitro* and *in vivo* experimentation of selected medicinal plants. Results revealed that there were significant ($p<0.05$) differences among the studied plants extract and lowest spermicidal activity was observed on incubating the semen with ethanolic extract of *P. tuberosa* tested at different time intervals and normal saline as control. Additionally, it was stated that the motility of the spermatozoa in control-treated semen did not significantly ($p>0.05$) decrease. It was also reported that incubation of the semen with the extracts of *P. tuberosa* enhanced the motility and preserved the viability of spermatozoa (Figure-6, 7 & 8).

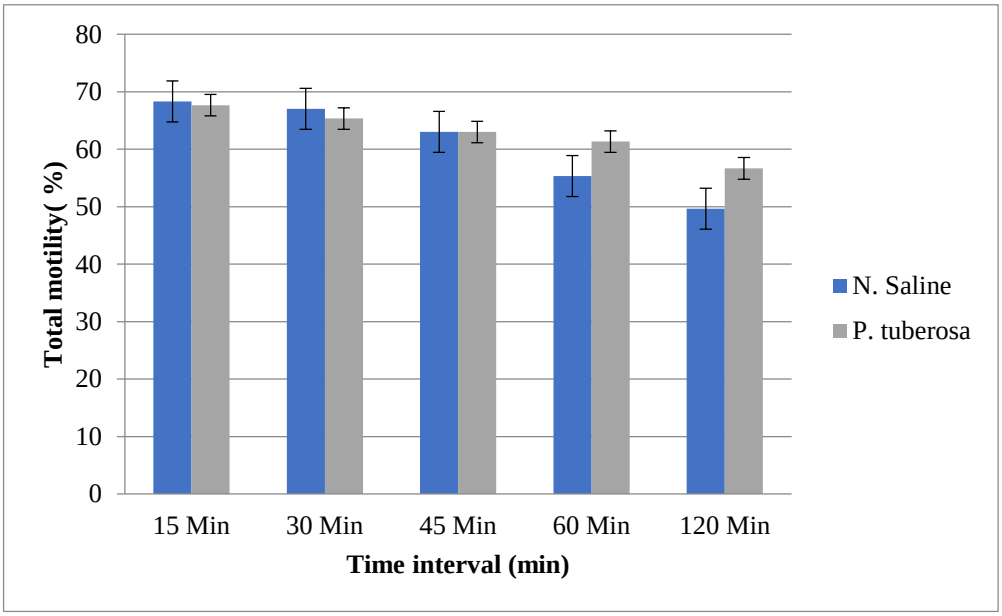

Figure-6 *In vitro* spermicidal activities evaluated by Total motility (%) of Spermatozoa of selected medicinal plant extract and control treatments at different time interval.

The values are mean ± SD of replicate determinations and bars share different alphabets are significantly ($P<0.05$).

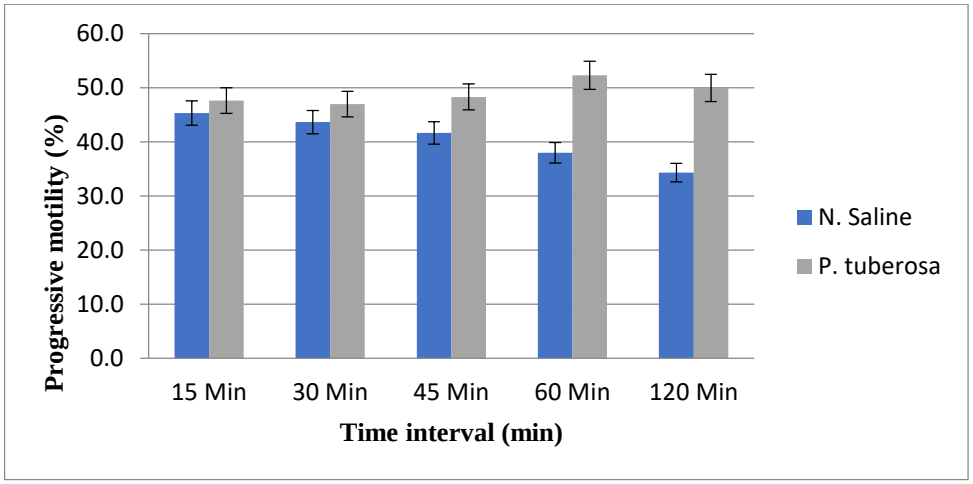

Figure-7 *In vitro* spermicidal activities evaluated by Progressive Motility (%) of Spermatozoa of selected medicinal plant extract and control treatments at different time interval. The values are mean ± SD of replicate determinations and bars share different alphabets are significantly (P<0.05).

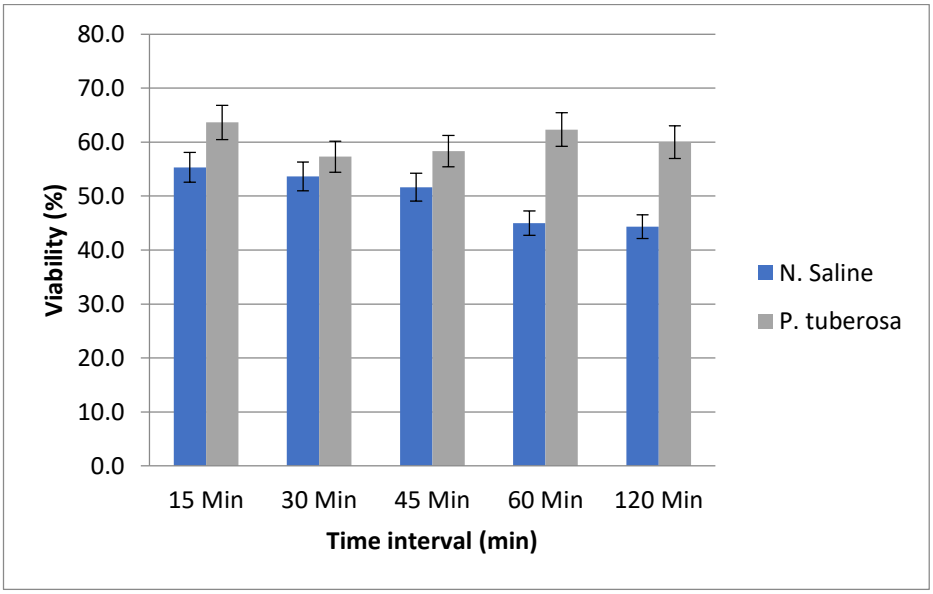

Figure-8 *In vitro* spermicidal activity evaluated by Viability (%) of Spermatozoa of selected medicinal plant extracts and control treatments at different time interval.

The values are mean ± SD of replicate determinations and bars share different alphabets are significantly (P<0.05).

Discussion

Medicinal plants have proven to possess traditional and scientifically proven aphrodisiac effects that can enhance passion, libido and sexual performance(R. Singh et al., 2013). Various ethnobotanical surveys have characterized a large number of plants traditionally used as aphrodisiacs but only a few of them are scientifically validated for the treatment of male sexual dysfunction. Several plants species have great impact on quality and quantity of sperms. This article with phytochemical analysis mainly enumerated the aphrodisiac potential of *P. tuberosa* and the remarkable results were found on sperms health. During in vivo study, similar results were also found of methanolic extract of *C. zambesicus* at the dose of 5-10mg/kg increased number of spermatozoa and its motility(Ofusori et al., 2007).

It was widely acknowledged that the alkaloid found in medicinal plants were responsible for the therapeutic uses, such as analgesic, antibacterial, and antispasmodic (Okwu & Iroabuchi, 2009).

Similarly phenolic contents are responsible for the significant antioxidant potential (Lu, Yuan, Zeng, & Chen, 2011) It was also reported that polar solvents like ethanol and methanol have capacity to dissolve out the antioxidant phenolic compounds from medicinal plants (Esmaeili, Hashemiravan, Eshaghi, & Gandomi, 2021). The most significant natural phenolics agents include flavonoids, which act as natural antioxidants (Gutiérrez-del-Río et al., 2021). Results showed these natural flavonoids, which also have the potential to prevent the oxidation of low-density lipoproteins, are responsible for the reactive oxygen species detoxifying capacity (Chen, Wang, Zhu, Xiao, & Zhang, 2018).The tubers of *P. tuberosa* contain a variety of pharmacologically significant compounds, includingreducing sugars monosaccharides, pentox sugars, hexose sugars, proteins, steroids, cardiac glycosides, anthroquine glycosides, saponin glycosides, cyanogenetic glycosides, coumarin glycosides, flavonoids, alkaloids and tannins(Satpathy et al., 2021). *P. tuberos*a acoholic extract possess number of phytochemicals namely puerarin, genistein, quercetin, irisolidone, diadzein and mangferin etc. (Shilpashree, Dang, & Das, 2015).Results of this study regarding total soluble proteins are supported by the findings of (Kanthaliya, Joshi, Arora, Alqahtani, & Abd_Allah, 2023) that *P. tuberosa* have considerable concentration of proteins contents.

There are different minerals like manganese copper and zinc which have incredible role infertility and sperm health. This research was also first time performed the mineral analysis in medicinal plants and found that heavy metals are found in negligible concentrations. While considerable concentration of elements having positive role in the spermatogenesis and fertility were identified in *P. tuberosa*. Quantitative analysis shows a clear link between total phenolic and total flavonoid chemicals. According to research, the capacity of hydroxylation and structural stability are the parameters which determine the radical scavenging test response which is probably based on the amount of antioxidant phytochemicals found in under study plant extract. (Woldegiorgis, Abate, Haki, & Ziegler, 2014).

According to the findings of this study *P. tuberosa* extract showed the significant antioxidant activity and may be used as a natural antioxidant in the development of medicinal preparations, food additives, and cosmetics. Previous study also supports our results that *P. tuberosa* showed notable potential to neutralize free radicals such the DPPH, ABTS.+ radical, and nitric oxide. *P. tuberose* along with other wide range of pharmacological effects, also have potential as antioxidant agent due to the detoxification of free radicals. The results of our study concur with those of (Bharti et al., 2021), who found that the *P. tuberosa* root extract had significant ferric ions (Fe3+) lowering capacity. Due to the presence of considerable amount of phenol and flavones it shows significant antioxidant activity. *P. tuberosa* reduce oxidative stress which ultimately reduce risk of various diseases like heart attack, atherosclerosis, various age-related disorders and stroke.

P. tuberose also have significant ($p<0.05$) membrane stabilizing (antihemolytic) potential and it could be concluded that *P. tuberosa* extract showed the low toxic activity. According to study result it is safe and can be used for further studies on animals for the development of therapeutic modality for human. The strong antioxidant capacity of several therapeutic plants may explain these benefits. Additionally, several medicinal plants have become important in the treatment of infertility issues due to the presence of phytochemical components and enough antioxidant minerals. It was the first time according to literature review; this medicinal plant was tested for spermicidal activities. *P. tuberosa* extract in albino rats was found to be have remarkable aphrodisiac activity and dose dependent increase in sexual behavior along with positive findings regarding histological architecture and weight of sexual organs were also observed (Chauhan, Sharma, Thakur, Christine Helena Frankland Sawaya, & Dixit, 2013). The findings are in the favor to use selected medicinal plant *P. tuberosa* as is potential agents in the treatment of abnormal spermatogenesis and in teretozoospermia conditions. Moreover, such findings also revealed that these medicinal plants could be potent candidates for the preservation of spermatozoa during artificial intrauterine insemination (IUI) and in-vitro fertilization (IVF).

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