EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 1
Issue: 3
609-614
Year: 2014

ASSESSMENT OF THE TOXICITY OF ACETONE EXTRACT OF *GOSSYPIUM HIRSUTUM* LEAF ON HUMAN SPERM CELLS

Nwobodo H.A.*

Department of Medical Microbiology, College of Medicine, Enugu State University of Science & Technology, Enugu, Enugu State, Nigeria.

***Corresponding Author: Nwobodo H.A.**
Department of Medical Microbiology, College of Medicine, Enugu State University of Science & Technology, Enugu, Enugu State, Nigeria.

Article Received on 10/09/2014 **Article Revised on 04/10/2014** **Article Accepted on 28/10/2014**

ABSTRACT
Research has shown that *Gossypium hirsutum* has inhibitory effects on cancer cell lines, hence the use of its leaf, root and bark by the locals in cancer treatment. Acetone leaf extract of *Gossypium hirsutum* was evaluated for toxicity on human sperm cells using standard methods. The leaves of the plant were processed and the phytochemical and toxicity on human sperm cells analyzed using standard methods. The extract were added at different time intervals after sperm production (0mins, 45mins, 90mins, 135mins, 180mins, 225mins and 270mins) and at different concentrations (50 mg/ml, 23 mg/ml, 12.5mg/m 6.25mg/ml). The result showed variations in the motility of the sperms cells and viable sperm cell count at the various concentrations of the acetone leaf extract of *Gossypium hirsutum* used. The result showed that as the concentration of the leaf extract increased, motility and viable count decreased. Normal motility of the sperm cells and viable sperm cell count was recorded at 20mg/ml, 70mg/ml, 85mg/l, 100mg/ml, 119mg/ml at 45mins. The result showed no motility and viability at 270mins. Hence, the research suggested that acetone leaf extract of *Gossypium hirsutum* has toxic effect on human sperm cells. Further research should be conducted to isolate, purify and characterize the bioactive component of the leaf extract of *G. hirsutum* extract responsible for the observed toxic effect.

KEYWORDS: Toxicity, Acetone leaf extract, *Gossypium hirsutum*, sperm cells.

INTRODUCTION
Gossypium hirsutum, known as upland cotton or Mexican cotton, is the most widely planted species of cotton in the United States, constituting some 95% of all cotton production and is native to Central America and possibly Mexico (Khaleequr, *et. al.*, 2012). Worldwide; the figure is about 90% of all production for this species (Sistler, 1981).

Gossypium hirsutum, includes a number of varieties or cross-bred cultivars with varying fiber lengths and tolerances to a number of growing conditions (Khaleequr, *et. al.*, 2012). The longer length varieties are called "Long Staple Upland" and the shorter length varieties are referred to as "Short Staple Upland" (Bijaj, 1998).. The long staple varieties are the most widely cultivated in commercial production (Parnell, 1981).

Gossypol [1,1',6,6',7,7,-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2'-binaphathalene)-8,8'-dicarboxaldehyde] is adi-sesquiterpene produced by species in the genus *Gossypium* (family Malvaceae) (Smith 1961). The pigment, localized in lysogenous glands distributed in the epidermal layer of most tissues, confers insect resistance and serves as a deterrent to other herbivores (Smith 1961). High levels of gossypol are also localized in the epidermal and cortex tissues of cotton roots (Pigg *et al*., 1941; Mace *et al.*, 1974) from which gossypol may be secreted extracellularly (Hunter *et al* 1978). The medicinal properties of *Gossypium hirsutum* are widely recognized in China, Africa, and India and in folk medicine practices of the southern USA. Low fertility rates in China in regions where crude cotton seed oil was used for human consumption led to the discovery of gossypol as a male oral contraceptive (Anonymous, 1997 and Anonymous, 2007).

Gossypium hirsutum root bark contains acidic resin, volatile oil, linoleic-acid, phenolic-acid, ceryl-alcohol, oleic and palmitic acids, isoquercitrin, quercimcritin, qucrcetin-3 -glucoside, hirsutrin, serotonin, gossypicyanin, histamine and other compounds (Duke, 1983.). Seed contain gossypol. Several research groups have reported the inhibitory effects of *Gossypium hirsutum* and related compounds on in vitro growth of cancer cell lines (Band *et al.* 1989; Le Blanc *et al.*, 2002).

Does *Gossypium hirsutum* have toxic effect on cells? Given the acclaimed benefits of this plant for medical and other applications, this research sought to analyze the toxicity of acetone extract of *Gossypium hirsutum* leaf on human sperm cells.

OBJECTIVE OF THE RESEARCH

I. To carry out solvent extraction of *G. hirsutum* leaf using acetone.
II. To determine the phytochemical composition of *G. hirsutum* leaf.
III. To test different concentrations of acetone extract of *G. hirsutum* leaf on sperm cells.

MATERIALS AND METHOD

Materials used for the research include: Incubator (model no DNP-9022A), Microscope (model: main power-210-230v/50-60Hz, Lamp 6v/120w Halogen), Weighing Balance (model: Scout Pro Spu600l by Ohaus corporation, Pine brook, NJ USA), Neauber chamber, acetone- GPR (Vickers Laboratories Ltd) and Buffered saline (Sodium bicarbonate ($NaHCO_3$)- 5g, Formalin (Neutral)- 1ml, Distilled water- 100mI).

Collection of samples

Fresh *G. hirsutum* leaves were collected from Obuoffia Awkunanaw in Nkanu West Local Government Area of Enugu State during the month of June, 2011. The leaves were identified by a trained plant taxonomist Prof J.C Okafor of the Department of Applied Biology and Biotechnology, Enugu State University of Science and Technology (ESUT), Enugu, Nigeria.

Processing of plant samples

The fresh leaves collected were screened. Those becoming yellowish, dried or affected by disease were removed and only the greenish leaves without spot were used. The leaves were washed with tap water and rinsed in sterile distilled water, spread on a local tray made of palm fronds to enhance draining of water. The tray was kept inside a well ventilated room and the leaves turned after every 6hrs to that enhance even drying. After 2 weeks, the leaves became friable and were ground into powder using clean mortar and pestle bought for the purpose of the research. The powder was stored in air tight dark sterile reagent bottles until processed further.

Extraction of plant materials

Extraction of plant material was carried out following the method described by Egereonu and Mokwe (2005). Forty (40) gram of the powder was extracted in 250ml of acetone using maceration method in a conical flask. The flask was corked using aluminum foil, shaken six (6) hourly for 24 hours and filtration carried out using muslin cloth. The filtrate was concentrated by evaporation using water bath at 37^{o}C for one hour and further air-dried at room temperature to get crude acetone leaf extract of *G. hirsutum*.

Phytochemical screening

Phytochemical analysis of G. hirsutum followed the method described by Okerulu and Ani (2001) and Harborne (1973).

Test for the presence of alkaloids

One (1) gram of the acetone leaf extract of *G. hirsutum* was boiled with 25rn1 of 2% HCI on a water bath for 5 minutes. The cooled mixture was filtered and 1ml portion of the filtrate treated with 2 drops of the following reagents:

- Dragendorff's reagent (Bismuth in potassium iodide solution). A red precipitate indicates the presence of alkaloid.
- Mayer's reagent (potassium in mercuric iodide solution). A creamy white coloured precipitate indicates the presence of alkaloids.
- Wagner's reagent (iodine in potassium iodide solution). A reddish brown coloured precipitate indicates the presence of alkaloid.

Test for the presence of flavonoids

Half (1/2) gram of the powder was heated with 10ml of ethylacetate in a water bath for 3 minutes. The mixture was filtered and the filtrate used for the following tests:

- Four (4) m1 of the filtrate was shaken with 1ml of 1% aluminum chloride solution and observed for light yellow colouration in the ethyl acetate layer. A yellow colouration in the ethylacetate layer indicates the presence of flavonoids.
- Four (4) m1 of the filtrate was shaken with 1ml diluted ammonia. The layers were allowed to separate. A yellow colouration at the ammonia layer indicates the presence of flavonoids.

Test for glycosides

About 2.5m1 of dilute H_2SO_4 acid was added to 5ml of each extract in a test tube and boiled on a water bath for 1 5mm. This was cooled and neutralized with 20% KOH solution. 5m1 of a mixture of Fehling's solution A and B was added and boiled. A brick-red precipitate shows reducing sugars released as a result of the hydrolysis

Test for the presence of saponins

Half (1/2) gram of the powder was boiled with l0ml of distilled water for 5minutes. The mixture was filtered while still hot. The filtrate was then used for the following tests.

I. Emulsion test

One (1) ml of the filtrate was added to 2 drops of olive oil. The mixture was shaken and observed for the formation of emulsion.

II. Frothing test

One (1) ml of the filtrate was diluted with 4rn1 of distilled water. The mixture was shaken vigorously and then observed on standing for stable froth.

Test for the presence of tannins
One (1) gram of the powder was boiled with 5ml of 45% ethanol for 5 minutes. The mixture was cooled and filtered. The filtrate was used for the following tests:

Test for steroids
To I ml of the powder in a test tube was 5 drops of concentrated H_2SO_4 added**.** A red colouration indicates the presence of steroids.

Preparation of different concentrations of the extract
One hundred (100) mg/ml stock acetone extract was prepared by dissolving 1g of powder in 10 ml normal saline. From the stock, different concentrations (50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml using doubling dilution method of 1:2, 1:4, 1:8, and 1:16 respectively.

- -

Preparation of Sperm cells
Fresh semen sample was donated by a volunteer and immediately analyzed (Cheesbrough, 2000 and Bjorndahl *et. al.*, 2004).

Sperm count
Cell count was performed on the semen with a counting chamber (haemocytometcr). The semen was diluted 1:20 in a test tube with buffered saline using a pipette. The haemocytometer was charged, the chamber filled with the diluted semen and cells focused using x10 objective lens and counted using x40 objective lens. The buffered saline immobilizes and preserves the sperm cells.

Counting procedure
To calculate the number of spermatozoa per ml counted in the chamber, a multiplication factor was used. The multiplication factor for square 5 is 10,000 for all large squares 1-5, the factor is 2000, for the smaller square 5a, b, c, d and e, the multiplication factor is 50,000

Calculations
No of sperm cells counted = n
Multiplication factor = 50,000
Dilution factor = 20
Sperm count per ml = n x 50,000 x 20 = n x 10^6 (sperm cell count)
Total sperm count = n x 10^6 x volume of semen
= n x 10^6 x volume per ejaculation

Toxicity test
Two drops of the cells (1:20 dilution of the semen) were placed in microtitre wells. This was followed by the addition of 2 drops of the various dilutions. After gentle rocking, motility and cell count was carried out at intervals of 0mins, 45mins, 90rnins, l35mins, l80mins, 225mins and 270mins. A drop of Eosin-Nigrosin was added to each well before counting. Results were recorded (Viable cells resist being stained, while dead cells are stained) (Bjorndahl *et. al.*, 2004).

RESULTS
Phytochemical analysis
Phytochemical analysis of the leaves of *Gossypium hirsutum* using acetone showed that saponins, tannins, alkaloids and flavonoids were present, while steroids was absent as presented in table 1.

Table 1: Phytochemical of Gossypium hirsutum leaves.

Photochemical	Estimated concentration
Alkaloids	+++
Flavonoids	+
Saponins	+++
Glycosides	++
Steriods	-
Tannins	+++

Key
Absent
\+ - Scanty
++ - Moderate
+++ - Abundant

Seminalysis
The semen was moderately viscous. Morphologically normal cells were 90% against 10% abnormal cells. Majority of the cells (90%) were actively motile, 5% sluggishly motile and 5% non-motile. Viable cell count was 122 x 10^6.
Time produced : 2:20pm
Time analyzed : 2:40pm
Colour : milkfish/grayish
Viscosity : moderately viscous

Morphology
Normal- 90%
Abnormal- 10%

Motility
Non-motile: 5%
Sluggish cells: 10%
Active motile cells: 83%
Viable cell count: 122 x 10^6 cells/ml

Toxicity test
The extract had varying effects on the motility of sperm cells depending on the time. At the time- 0 min of the experiment, motility was high against the various concentrations except among the negative controls where no motility was recorded. As the interval between the addition of the extract increased, motility of the sperm cells decreased, rate of decrease increasing with increase in concentration of the extract as shown in table 2.

Table 2: Effect of various concentrations of acetone extract of *G. hirsutum* on motility of sperm cells.

S/N	Extract Dilution	Extract Conc (Mg/ml)	Time (Mins)						
			0	45	90	135	180	225	270
1	Neat	100	++	-	-	-	-	-	-
2	1:2	50.0	+++	+	-	-	-	-	-
3	1:4	25.0	+++	+	+	-	-	-	-
4	1:8	12.5	+++	+	+	-	-	-	-
5	1:16	6.25	+++	++	+	+	-	-	-
6	Negative Controls	Semen Diluent	-	-	-	-	-	-	-
7	Positive Controls	Water	+++	+++	++	+	+	+	-

Keys
Negative control: 1:2 dilution of semen to water (i.e. 1 drop of semen: 19 drops of distilled water)
Positive control: 1 drop of semen: 19 drops of buffered saline
- : No Motility (<1%)
+ : Slightly Motility (1-49%)
++ : Moderately Motile (1-49%)
+++ : Highly Motile (70% and above).

Table 3: Toxic effect of various concentrations of acetone extract of *G. hirsutum* on viable sperm cells count.

S/N	Extract Dilution	Extract Conc (Mg/ml)	Cell count (n x 106 cells/ml)/ Time (Mins)						
			0	45	90	135	180	225	270
1	Neat	100	122	20	-	-	-	-	-
2	1:2	50.0	122	70	50	37	15	-	-
3	1:4	25.0	122	85	61	49	24	-	-
4	1:8	12.5	122	100	89	64	33	12	-
5	1:16	6.25	122	119	105	99	67	39	19
6	Negative Controls	-	-	-	-	-	-	-	-
7	Positive Controls	122	122	118	116	92	87	49	13

Keys
Negative control: 1:20 dilution of semen to water (i. e. 1 drop of semen: 19 drops of distilled water).
Positive control: 1 drop of semen: 19 drops of buffered saline.

DISCUSSION

Phytochemical analysis of acetone leaf extract of *Gossypium hirsutum* showed that saponins, tannins, alkaloids were abundant (+++), glycosides were moderate (++), flavonoids were scanty (+), while steroids were absent (-) as could be seen from the result in table 1. The observed toxic effect of the extract may be attributed to single or combined effect of the phytochemical which form the bioactive constituents of the leaf. This is an agreement with the finding of Gupta and Sharma (2006) in a review on medicinal plants exhibiting anti-fertility activity in males.

Seminalysis of semen sample used showed that the viable count of 122 x 10^6 cells/ml for the study was within the normal sperm cell count range. In a study titled 'Why the WHO Recommendations for Eosin-Nigrosin Staining Techniques for Human Sperm Vitality Assessment Must Change' the normal sperm cell count range of humans is 50-150million/ml (Bjorndahl *et. al.*, 2004).

The toxic effect of acetone leaf extract of *Gossypium hirsutum* on motility of sperm cells carried out using different concentrations of the extracts (50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml) at different time intervals measured in minutes (0, 45, 90, l35, 180, 225 and 270) as shown in table 2 revealed that toxic effect of the extract was concentration and time dependent. The result indicated a gradual decrease in the motility of the sperm cell as the concentration of the extract increased. When the concentration was 50mg/ml, there was no motility at 45mins, 90mins, l35mins, 180mins, 225mm and 270mins. At 25mg/ml motility decreased when compared with that at the concentration of 12.5 mg/ml and 6.25 mg/ml across the time interval of 45mins, 90mins at which effect of the extract was measured. No motility was observed at l35mins, 225mins and 270mins at 25 mg/ml. As the concentration was lowered to 6.25mg/ml; motility of sperm cells at 45mins, 90mins, l35mins, and l80mins was normal and compared with the negative control. However, no motility was observed at 225mins and 270mins using the lowest concentration of the extract (6.25 mg/ml). This finding is in agreement with that reported by Kabiruddin and Makhzul (2007) in India showing that *Gossypium hirsutum* has activity against sperm cells. The activity according to this research could be anti-motility leading to reduction in sperm cell motility. Hence, it could be deduced from the study that acetone extract of *Gossypium hirsutum* affect and cause reduction in motility of sperm cells. This may be the scientific basis for its use as male contraceptive among some local folks and accounts for the compound antifertility actions (Gupta and Sharma, 2006). Thus,

consumption of the extract of *G. hirsutum* could impair the physiology of sperm cells.

The effect of acetone leaf extract of *Gossypium hirsutum* on viable sperm cell count carried out using different concentrations (100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6 mg/ml at different time intervals (0 min, 45mins, 90mins, l35mins, l80mins, 225mins and 270mins) as shown in table 3, showed gradual decrease in sperm cell count as the extract concentration increased. At 100mg/ml there were viable sperm cells (20×10^6) at 45mins, with complete loss of viability at 90mins, l35mins, 225mins and 270mins respectively. At 50mg/ml, sperm cell viability at 45min, 90min, 135min, 180mins were 70×10^6, 50×10^6, 37×10^6 and 15×10^6 cells/ml respectively. No viability was recorded at the same concentration of 50 mg/ml in 225mins and 270mins. As the concentration reduced to 6.25 mg/ml, viable sperm cell counts at 45mins, 90rnins, l35mins, 225mins, 225mins, 270rnins were 119×10^6, 105×10^6, 99×10^6, 99×10^6, 67×10^6, 39×10^6 and 19×10^6 cells/ml respectively. Reduction in viable cell as concentration and time increased suggests that the extract may have concentration-Time dependent toxic effect on sperm cells. The finding of this research is in consonance with that in a study on human sperm nuclear protein after doses (15 mg/ml for 12 weeks and 10 mg/ml for 32 weeks) of gossypol (*G. hirsutum* cotton seed extract) was administered. Administration of gossypol as stated above led to infertility (Van Poznak *et. al*., 2001., Tso, 1984) and also with the findings of Gupta and Sharma (2006) in which Gossypium demonstrated activity against sperm cells.

CONCLUSION

Gossypium hirsutum has numerous medicinal effects. These beneficial effects notwithstanding, care should be exercised in its use especially among males since the extract could have toxic effect on sperm cells as revealed by this research. Further research should be conducted to isolate, purify and characterize the active ingredients responsible for the observed toxic effect of acetone leaf extract of *G. hirsutum* on human sperm cells

REFERENCES

1. Anonymous.1997. Standardization of single drugs of Unani medicine. Part III. New Delhi: Central Council of Research in Unani Medicine, 1997; 229-34.
2. Anonymous.2007. The Unani Pharmacopoeia of India. Part 1, Vol.1. New Delhi: Dept. of Yurveda, Yoga, Unani, Siddha and Homeopathy, 2007; 66-7.
3. Band V.; Hoffer A. P.; Band H.; Rhinchardt A. I Knapp R. C.; Matlin S. A.; Anderson D .J. Antiproliferative effect of gossypol and its optical isomers on human reproductive cancer cell lines. Gynecol. Oncol, 1989; 32: 273-277.
4. Bjorndahl, L., Soderlund, I., Johansson., Mohammadieh, M., Pourian, M. R. and Kvist, U. Why the WHO Recommendations for Eosin-Nigrosin Staining Techniques for Human Sperm Vitality Assessment Must Change. Andrology, 2004; 25(5): 671 -678.
5. Cheesbrough, M. *District laboratory practice in Tropical Countries.* Part 2. Cambridge University Press. U.K., 2000.
6. Duke, J. A. Handbook of Energy Crops. Unpublished, 1983.
7. Egereonu, U. U., Mokwe, N. R., Characterization of the Extract from *Bryophyllum pinnatum* Leaves. Journal of Chemical Society of Nigeria, 2005; 30(2): 192–196.
8. Gupta, R. S, and Sharma, R. A. Review on Medicinal Plants Exhibiting Anti-Fertility Activity in Males. Natural Product Radiance, 2006; 5(5): 389–410.
9. Harborne, J. B. Phytochemical Methods A Guide to Modern Techniques of Plant Analysis. Springer Netherlands, 1973; 182–201.
10. Hunter R. E.; Halloin J. M.; Veech J. A Carter W. W. Exudation of terpenoids by cotton roots. Plant Soil, 1978; 50: 237-240.
11. Kabiruddin M. and Makhzul M. New Delhi: Idarae Kitabus Shifa, 2007; 136–7.
12. Khaleequr, R., Arshiya, S., Shafeequr, R. *Gossypium herbaceum* Linn: An Ethnopharmacological Review. Journal of Pharmaceutical and Scientific Innovation, 2012; 1(5): 1-5.
13. Le Blanc M.; Russo J.; Kudelka A. P.; Smith.1. A. An in vitro study of inhibitory activity of Gossypol, a cottonseed extract, in human carcinoma cell lines. Pharmacol. Res, 2002; 46: 551-555.
14. Ojha, P., Dhar, J. D., Dwivedi, A. K., Singh, R. L., Gupta, G. Effect of Ant-spermatogenic Agents on cell Marker Enzymes of Rat Sertoli cells in vitro Contraception. Journal of Pharmaceutical and Scientific Innovation, 2006; 73: 102–106.
15. Okerulu, I. O., Ani, C. J., The phytochemical Analysis and Antibacterial Screening of Extracts of *Tetracarpidium conophorum*. Journal of Chemical Society of Nigeria, 2001; 26(1): 53-55.
16. Okwori, A. E. J., Dina, C. O., Junaids, S., Okeke, I. O., Adetunji, J. A., Olabode, A. O. Antibacterial Activities of *Ageratum conyzoides* Extracts on selected Bacterial Pathogens. The Internet Journal of Microbiology, 2007; 4(1): 1-13.
17. Parnell, C.B., Jr. Cotton *Gossypium hirsutum*. In: McClure, T.A. and Lipinsky, E.S. (eds.), CRC handbook of biosolar resources. Resource materials. CRC Press, Inc., Boca Raton, FL, 1981; 2: 115–122.
18. Pigg, D. Cottonseed bread a new use for cotton. Texas Ag. Progress. Winter, 1980; 20.
19. Sistler, F.E. and Smith, P.A. A total energy model for cotton production. Louisiana Ag, 1981; 24(4): 22-23.
20. Tso, W. W. Gossypol inhibits Ehrlich ascites tumor cell proliferation. Cancer Lett, 1984; 24: 257.
21. Van Poznak, C., Seidman, A. D., Reidenberg, M. M., Moasser, M. M., Sklarin, N., Van Zee, K., Borgen, P., Gollub, M., Bacotti, D., Yao, T. J.,

Bloch, R., Ligueros, M., Sonenberg, M., Norton, L., and Hudis, C. Oral gossypol in the treatment of patients with refractory metastatic breast cancer: a phase I/II clinical trial. Breast Cancer res Treat, 2001; 66(3): 239-48.