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Cytotoxicity analysis by MTT assay of isolated Gossypol from Bt and Non-Bt Cotton Seeds on HeLa Cell Lines.

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ABSTRACT

The present paper deals with study of In-vitro cytotoxicity effect of isolated gossypol from Bt and Non-Bt cotton seeds on HeLa cell lines. Gossypol is a polyphenolic binaphthyl diadehyde natural yellow colored pigment. It is not only resistance substance for cotton plant's self – defense system against insect pests and possibly some diseases but also an important phytochemical compound of immense interest due to its several biological properties including anti-cancer,anti-HIV,anti-oxidation, antimicrobial and as male contraceptive. During this study gossypol exhibited broad spectrum of anti-cancer activity against the HeLa cell lines. The cytotoxicity effect of gossypol was detemined by MTT (3-(4-,5-dimethylthiazolyl-2)-2,5-dipheniltetrazolium bromide) assay. Gossypol from Bt and Non-Bt cotton seeds has shown dose dependent cytotoxicity effect against HeLa cell lines. In-vitro screening of the gossypol showed potential cytotoxic activity against HeLa cell lines. Mortality rate of 11.5884% and 22.6058% with 3µg/1µl concentration of isolated gossypol from Bt and Non-Bt cotton seed extracts was observed on HeLa cell lines respectively. Hence the inhibitory concentration at 50% (IC50) was fixed at 3µg/1µl of gossypol for HeLa cells. The standard anti-cancer drug Doxorubicin (1mg/ml) was also used in this study to confirm anti-cancer activity of gossypol isolated from Bt and Non-Bt cotton seed with 1.7828% cell viability. The present study confirms the mild toxic effect of gossypol on HeLa cell lines and can preferably be used as anti-cancerous drug in combination with other natural similar compounds to replace the synthetic chemical drugs for fewer side effects.

INTRODUCTION

Plants are the basic source of knowledge of modern medicine. Almost all the parts of the plant, viz, bark, root, stem and seeds are known to have various medicinal properties . The trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries and for the presence of antimicrobials. According to the world health organization (WHO), Medicinal plants would be the best source to obtain a variety of drugs. About 80% of individual medicines have compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency.The plant selected for this study is Cotton, the most important textile fiber crop and is the second most important oil crop in world. Cotton belongs to the family Malvaceae, genus *Gossypium* and comprises of 50 different species, of these only four species are cultivated in India.

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Gossypium arboreum and *Gossypium harbaceum* belongs to the old world diploid group, where as new world tetraploid cultivated species are *Gossypium hirsutum* and *Gossypium barbdance.*

In Andhra Pradesh cotton is cultivated in all the three different agro climatic regions viz., Coastal region, Telangana region and Rayalaseema region. Cotton seed which remains after cotton ginned is used to produce cotton seed oil. The plant has been found to possess several ethno medicinal uses. Cotton seeds and leaves feature in traditional medicine in various forms and are taken internally and applied externally to treat skin problems and injuries. For headaches, a drink is made from powdered cotton seeds and mixed with milk. Dysentery is also treated with an infusion of seeds and leaves. Spots and other skin conditions are treated using cotton seed or extracts from the leaves. In Western medicine, cotton is put to use in the form of dressings, bandages, swabs and cotton wool. Scientific investigations have shown that cotton roots and seeds contain certain compounds that may be beneficial to the health, potentially for treating Cancer and HIV. In India, Cervical Cancer is the most common cancer in women, even more common than breast cancer. Every year, in India 132,000 new cases are diagnosed and 74,000 women die due to this cancer .Cervical Cancer is caused by the Human Papilloma virus (HPV).(Source: WHO/ICO Information Centre on Human Papilloma Virus (HPV) and Cervical Cancer).

Cotton contain gossypol; a polyphenolic compound that is an integral part of the cotton plant's self-defence system against insect pests and possibly some diseases [8]. Some amount of gossypol tends to react with many natural substances in cottonseed and forms the bound gossypol that is non-harmful. However the unreacted gossypol known as "free gossypol" is toxic. Thus free gossypol is an anti-nutritional factor and Anti-cancer agent that limits the use of cottonseed and its products [7]. Gossypol [2, 2N-Bi (8-formyl-1, 6,7, trihydroxy5-isopropyl-3-methyl naphthalene)] is a crystalline compound. The molecular formula of gossypol is $C_{30}H_{30}O_8$.The inclusion compound formation by gossypol has been studied at different thermodynamic conditions. Most of the investigated molecules form more than one inclusion compound with gossypol. Polymorphism exhibited by gossypol inclusion compounds is dimorphism and trimorphism.Traditionally, the determination of cell growth is done by counting viable cells after staining with a vital dye.Trypan blue staining is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death) but the method is not sensitive and cannot be adapted for high throughput screening. Measuring the uptake of radioactive substances, usually tritium-labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells.

The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption max is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve [2]. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance. The use of the MTT method does have limitations influenced by: (1) the physiological state of cells and (2) variance in mitochondrial dehydrogenase activity in different cell types. Nevertheless, the MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves [1-20].

MATERIAL AND METHODS

Isolation and Detection of Gossypol

For extraction of gossypol three grams of Bt and Non-Bt cotton seed kernels obtained manually were crushed and extracted with diethyl ether (5 x 20 ml) [10]. The solvent was evaporated at low temperature till an oily material containing gossypol was obtained. This was stored for further use. Gossypol was extracted with aqueous acetone with the same method. The residual left after the extraction of free gossypol with aqueous acetone was soaked in 2M HCl solution (75 ml) for 10 min and then refluxed for 30 min. After cooling, the solution was filtered. The residue was washed with absolute ethanol (15ml).

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Then chloroform was evaporated from the extract at low temperature till an oily material containing gossypol was obtained. Specific chemical tests were performed for detection of gossypol in samples of seed extracts. For this purpose 5 ml of seed extract crude was dissolved in small volume of ethanol in 25 ml conical flask and final volume was made up to the mark by adding more ethanol. Two ml of each sample solution was taken in the test tube separately and equal amount of solid antimony chloride was added in each test tube and mixed thoroughly. Similar types of tests were performed with stannic chloride and lead acetate.

Table 1: Cytotoxic activity against the HeLa cancer cell lines

S.No	COMPOUND	Absorbance			% of viability			% of Cytotoxicity		
		Con.c (1µg)	Con.c (2µg)	Con.c (3µgl)	Con.c (1µg)	Con.c (2µg)	Con.c (3µg)	Con.c (1µg)	Con.c (2µg)	Con.c (3µg)
1	DMSO	0.5102	0.3669	0.3415	100	100	100	-	-	-
2	Doxorubicin	0.1879	0.1611	0.0161	36.836	43.909	49.25	0.1630	1.6828	1.7828
3	Non-Bt cotton seed isolated Gossypol	0.3711	0.3507	0.3207	81.218	95.576	97.23	18.7810	19.6058	22.6058
4	Bt cotton seed isolated Gossypol	0.4904	0.3353	0.2953	96.124	93.370	92.54	3.8757	9.5884	11.5884

Figure 1: Graphical diagram cytotoxic activity against the HeLa cancer cell lines

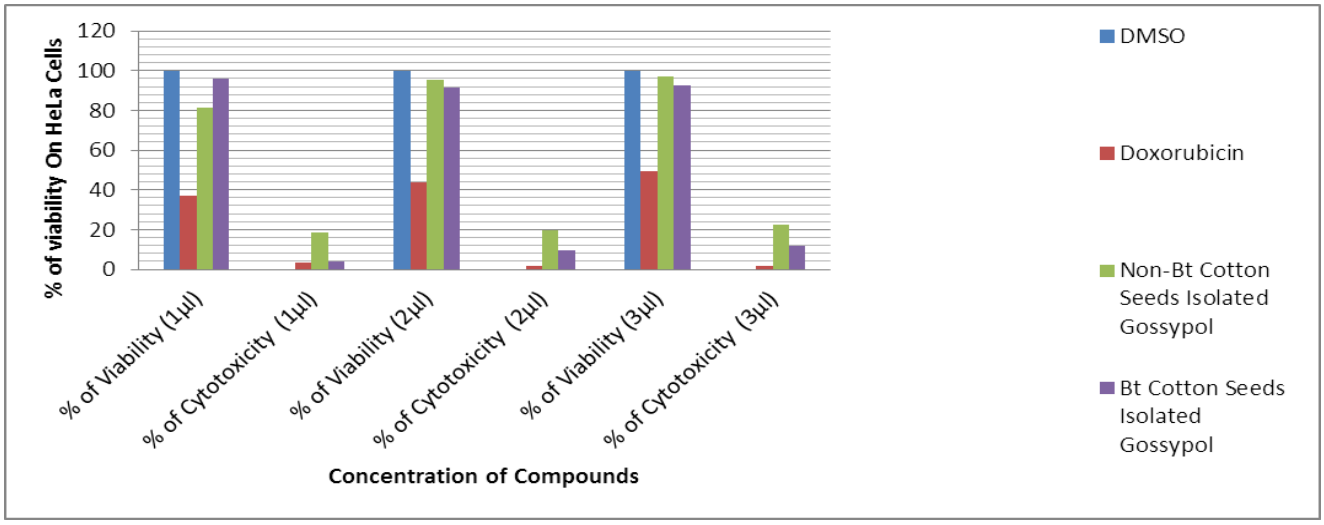

Table 2: The inhibitory concentration at 50% (IC50) was fixed at (Graph and Table)

3µg/ 1µl of Doxorubicin and Bt and Non-Bt cotton seeds isolated Gossypol for HeLa cells.

S.No	Compound	IC 50 values
1	Doxorubicin	1.7828
2	Non Bt(Gossypol)	22.6055
3	Bt(Gossypol)	11.5884

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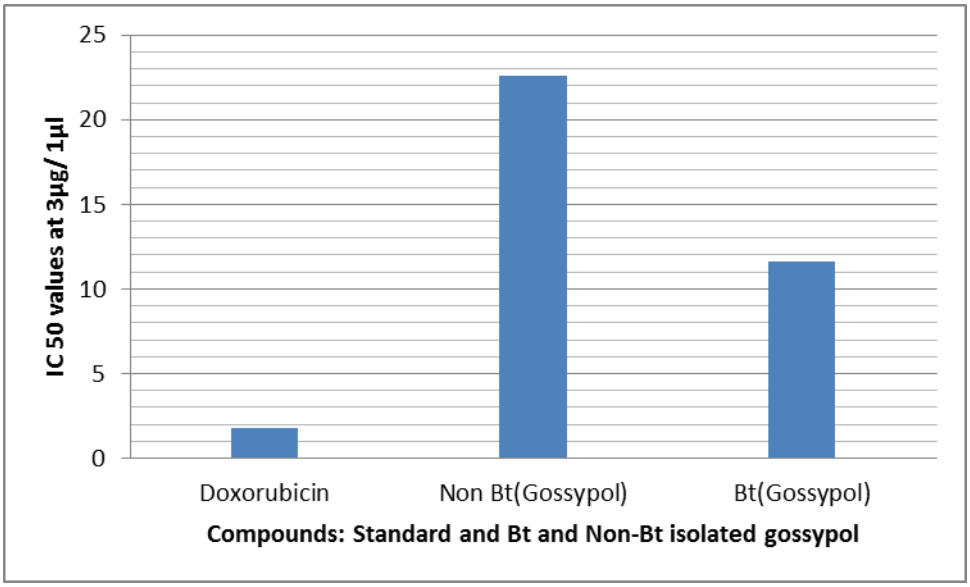

Figure 2

Evaluation of cytotoxic activity:

Cell viability and cytotoxicity assay were used for drug screening and cytotoxicity tests of chemicals. HeLa (Human cervix adeno carcinoma), cell lines were obtained from NCCS Pune. (HeLa cells were provided by Prof.Satyanarayana Singh Coordinator, DBT-ISLARE, CFRD Osmania University, Hyderabad).

MTT assay is the best known method for determining mitochondrial dehydrogenase activity in the living cells. In this method, MTT is reduced to a purple formazan by NADH. However, MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Therefore prior to measuring the absorbance, an organic solvent is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error (Alley,M.C.,et al 1988). HeLa cells were cultured in RPMI 1640 medium with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin. HeLa cell lines were sub cultured and were maintained at 37^{0}C at 5% CO_2 in CO_2 incubator. Cultures were continuously monitored every 24 hr. observed under an inverted microscope to assess the degree of confluency and to confirm the absence of any microorganism contaminants. In-vitro study of cytotoxicity effect of Bt and Non-Bt cotton seeds isolated Gossypol was assessed by MTT (3- (4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Cell lines were sub cultured and 250µl of media (containing 10000cells) were transferred into 96 well plates and incubated for 24 hr. The cells were subcultured with 100µl of fresh medium. Isolated Gossypol was added at different concentration (1-3µg/µl) and then final volume was made to 200µl with the media and incubated for 4 hr. After incubation media containing drug was removed. 20µl of MTT reagent (6mg/ml in PBS) was added to each well containing media and incubated for 3 hr at 37 °C under an atmosphere of 5% CO_2 until a purple precipitate was observed. Media was removed without disturbing cells and 200µl DMSO (MTT solvent) was added to dissolve the purple precipitate. Absorbance was read at 570 nm with a reference filter of 630 nm. Percentage cytotoxicity was calculated and used for finding the IC50 value of the concentration required for 50% cell death by Gossypol, isolated from Bt and Non-Bt cotton seed.

RESULTS AND DISCUSION

For detection of gossypol in the samples of Bt and Non-Bt cottonseed extracts three specific chemical conformation tests with $SbCl_3$, $Pb(CH_3COO)_2$ and $SnCl_3$ were performed. Turbid reddish complex appeared in case of $SbCl_3$ after 15 min. While reddish precipitate appeared after 10 minutes with Stannic chloride. Test with lead acetate gave yellowish precipitate that appeared after 20 min confirming presence of gossypol in the samples. In this study we have employed a dose dependent approach to evaluate the toxicity of the isolated gossypol from both Bt and non-Bt cotton seeds on HeLa cell lines at different concentration (1µg/1µl -3µg/1µl).

Results revealed that there is a significance cytotoxicity observed with dose dependent concentration of Bt and non-Bt cotton seed isolated gossypol like 1µg/1µl,2µg/1µl and 3µg/1µl respectively on HeLa cell lines (Table 1 and Fig 1). Doxorubicin is used for standard anti cancerous drug. From the result it is confirmed that DMSO has no effect on HeLa cells and is used as dissolving solvent for gossypol. At 1µg/1µl of non-Bt and Bt gossypol dose the viability and cytotoxicity percentage of HeLa cell lines is 81.21%, 93.37%, 18.78% and 3.87% respectively. Similarly at 2µg/1µl of Non-Bt and Bt gossypol dose the viability and cytotoxicity percentage of HeLa cell lines is 95.57%, 93.37%, 19.6% and 9.58% respectively. At 3µg/1µl of Non-Bt and Bt gossypol dose the viability and cytotoxicity percentage of HeLa cell lines is 97.2%, 92.5%, 22.6% and 11.5%. According to our previous reports the levels of gossypol is more in non-Bt cotton seeds correspondingly the cytotoxicity was found to be more for non-Bt cotton seed isolated gossypol with 3µg/1µl dose dependent concentration.

Cytoxicity activity of gossypol on Human Breast cancer cells was reported by Jerzy w.Jarsozewski *et all*;(1990), Gossypol inhibited growth and apoptosis of Human Head and neck Squamous cell Carcinoma in vivo was reported by Keith G. wolter *et al* [9]. Increase in the percentage of apoptotic cells was observed in treated tumors versus control gossypol induced apoptosis in ovarian cancer cells through oxidative stress were reported by Romano *et al* [8]. In comparison to above three references, in this study also anticancerous activity of isolated gossypol from Bt and non Bt showed mild cytotoxicity on HeLa cancer cell lines. The in vitro screening of the Gossypol showed potential cytotoxic activity against the HeLa cancer cell lines and mortality rate of 11.5884 and 22.6058 % was observed in 3µg/1µl concentration of isolated gossypol from Bt and non Bt respectively. Hence the inhibitory concentration at 50% (IC50) was fixed at 3µg/1µl (Graph and table 2) of gossypol for HeLa cells [21-37].

CONCLUSION

Present analysis revealed more amount of gossypol in Non-Bt cotton seeds. Gossypol as a natural compound showed more percentage of cell viability compared to the standard anti-cancer drug Doxorubicin. Cytotoxicity for viable cells was maximum for non-Bt cotton seed gossypol indicating the effective control of HeLa cells over a period of time. The present study confirms the mild toxic effect of gossypol on HeLa cell lines and can preferably be used as anti-cancerous drug in combination with other natural similar compounds to replace the synthetic chemical drugs for fewer side effects.

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