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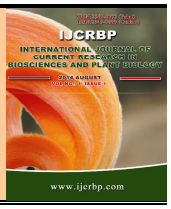


**Original Research Article**

# *Curculigo orchioides* Gaertn. Prevents Down Regulation of Actin and Tubulin Expression in Tests Induced by Carbendazim (MBC)

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**Abstract**

The work is to determine the toxic effect of the fungicide, carbendazim (MBC), methyl 2-benzamidazole carbamate on the gene for β-tubulin and β-actin and therefore the curative property of the *Curculigo orchioides* by performing the RT-PCR. The results of the gene expression by RT-PCR conforms that the genes β-tubulin and β-actin are affected by the fungicide as it is a microtubule as well as cytoskeletal poison and the abnormalities caused by the toxicity can be overcome by the curative property of the plant drug. The gene expression results have been quantified by using the software and graphs are plotted with the values drawn as results and they also suggest the same.

**Keywords**

Carbendazim
*Curculigo orchioides*
Fungicide
Gene expression
β-actin
β-tubulin

## Introduction

The studies in the field of the reproductive toxicity of carbendazim and the curative property of the *Curculigo orchioides* Gaertn. up to date has only spermatological studies but does not include gene expression results. The results confirm the facts once again through their results. The work had been carried out in the Wistar albino male rats. Presently, the data available for the same title ensures the toxic nature of the MBC and the phytotherapic effect of the plant drug. All the presently available data are spermatological studies and histopathological analysis results. It has been proved that the oral administration of the carbendazim produces reproductive toxicity as it is easily absorbed. As we are concerned about the oral administration, the previous works on the benomyl and MBC suggests that MBC is absorbed more readily from the gastrointestinal tract than its parent compound. This is a common property for the benomyl and its compounds. Exposure to toxicants early in development can lead to alterations that may affect reproductive function or performance well after the time of initial exposure (Gill et al., 1979; Giusti et al., 1995; Gray and Ostby, 1995).

Tubulin was long thought to be specific to eukaryotes. Microtubules can be characterized as long hollow cylinders with outer diameters between 20 nm and 30 nm and an inner diameter of about 14 nm. The main constituent of microtubules is the tubulin, which is a globular protein. In the native state, it exists as a heterodimer of an alpha and a beta form. Both forms have a molecular mass of approximately 50 kDa and a diameter of about 4 - 5 nm (Afshar at al., 1995; Marko et al., 1998). Actin participates in many important cellular processes including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, and the establishment and maintenance of cell junctions and cell shape. Many of these processes are mediated by extensive and intimate interactions of actin with cellular membranes (Doherty and McMahon, 2008; Vandekerckhove and Weber 1978). The fungicides have the capacity to bind to the important genes for the mitosis and sperm production. The MBC exhibits its fungicidal properties by binding to tubulin and totally disrupting the microtubule formation and mitosis. It is a burning issue that the toxicity should be overcome in a safe way. There comes to our rescue is the phytotherapy. The disruption of microtubules by the fungicide has been perfectly characterized by the peculiar disruptive effect seen in the spermatids such as broken heads, abnormal head shape, bending of tail etc. The microtubules are related to the gene β-tubulin and so the present work is concerned about that gene, its abnormalities after the administration of fungicide and the cured stage of the toxic effects by the plant drug. According to the earlier studies, the curative property of the plant drug used here is due to the chemical composition of the drug. (Mehta et al., 1983; 1990; 1991; Madhu Porwal et al., 1988).

## Materials and methods

### Animals

Male Wistar albino rats (90-100 days old and weighing 150 g to 200 g) were purchased from Annamalai University (Chidambaram, Tamil Nadu). The animals were housed 5 per cage in temperature (22º C) and humidity-controlled rooms (50±10%) rooms under 12-hr light, 12-hr darkness cycle. Animals were maintained on regular pelleted rat feed and tap water *ad libitum.* They were acclimated to housing conditions for at least 1 week prior to use.

### Chemicals and plant drug

Carbendazim was purchased from the local agro chemical supplier. Its trade name is Bavistin (BASF-India Ltd., Mumbai). Its IUPAC name is methyl 2-benzimidazole carbamate. Strength is 50%. The reagents for the total RNA isolation and RT-PCR were purchased from Genei Bangalore. The plant, *Curculigo orchioides* Gaertn. (Fig. 1), was collected from Bharathidasan University Campus, Tiruchirapalli. The tuberous roots were excised and washed with clean water. They were dried in the shade and grinded using the mechanical blender into a fine powder. The powder was then sieved using mesh the finest of the powder was collected.

### Primers

The primers used in the present study are β-actin and β-tubulin. They were bought from Genei, Bangalore.

### Administration of chemicals and plant drug

Based on the preliminary experiments, the following dose levels were selected. The oral administration was done by intubation. For the carbendazim, the powder was suspended in the olive oil with the dose level of 400 mg/kg. The chemical was given as single bolus dose. The dosage level for the plant drug was selected as 100mg/kg and 200 mg/kg. The plant drug was suspended in the milk and administrated by orally for 55 days. After the completion of administration period of the plant drug, the testis was used for the performance of RNA isolation and RT-PCR.

**Group-I** - Control
**Group-II** - MBC treatment
**Group-III** - *C. orchioides* 100mg/kg
**Group IV**- *C. orchioides* 200mg/kg

**Fig. 1: The plant, *Curculigo orchioides* used in the present study.**

**Isolation of Total RNA (Genei Total RNA Isolation Kit)**

Excise the tests tissue sample from the control and treated animals. The tissue samples were homogenized in the 600µl of lysis buffer. Centrifuge the lysate for 3 mins at 14,000rpm. Carefully remove the supernatant by pipetting and transfer it to a new microcentrifuge tube. Use only this supernatant for the subsequent steps. Add 1 volume of 70% ethanol to the cleared lysate and mix immediately by pipetting. Transfer upto 700µl of sample, including any precipitate that may have formed to an RNeasy spin column placed in a 2ml collection tube. Close the lid gently and centrifuge for 15sec at ≥ 8,000g (≥10,000rpm). Discard the flow-through. Reuse the collection tube for next step. Add 700µl buffer RW1 to an RNeasy spin column. Close the lid gently and centrifuge for 15sec at ≥ 8,000g (≥10,000rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube for next step. Add 500µl of RPE buffer to an RNeasy spin column. Close the lid gently and centrifuge for 2mins at ≥ 8,000g (≥10,000rpm) to wash the spin column membrane. Place the RNeasy spin column in a new 2 ml collection tube and discard the old collection tube with the flow-through. Close the lid gently and centrifuge at full speed for 1min. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30-50µl of RNase free water directly to the spin column membrane. Close the lid gently and centrifuge at full speed for 1min at ≥ 8,000g to elute the RNA.

Concentration of RNA= [40µg/ml X OD at 260 nm × dilution factor]/1000

**RT-PCR**
**Semi-quantitative RT-PCR**

Prepare Master Mix 1 containing the RNAsin, Genei 2X RT PCR reaction and primers (0.6 µM) and keep on ice. Master Mix 2 is prepared separately by denaturing Total RNA in RNAse free water and then added into each reaction sample individually. The final reaction volume is adjusted with RNase free water. Thaw the Genei 2X RT-PCR Reaction Mix. Place on ice. Setup Master Mix1 for the required number of reactions (plus one extra reaction) in a nuclease free microcentrifuge tube, mix properly. Centrifuge and place on ice. Dispense the required volume of the Master Mix 1 into each 0.2 ml PCR tube on ice. Setup Master Mix 2 [Template RNA+RNase free water; maximum 10 µl /50 µl reaction or 5 µl/ 25 µl reaction] in separate 0.2ml PCR tubes. Heat Master Mix 2

at 65ºC for 5 minutes to denature the template RNA. Add the desired volume of Master Mix 2 (denatured RNA) to the Master Mix 1into each PCR tube. Close the caps immediately. Mix well and spin down briefly to collect the reaction mixtures at the bottom of the tubes. Place the reactions in the thermal cycler equilibrated at the appropriate incubation temperature of the Reverse Transcription Reaction step and start the desired One Step RT-PCT program.

## Results

### Gene expression results

The gene expression results are quantified by the software Quantity One**.** The intensity and area of the bands are the data used by the software for the quantification procedure. The results can be determined by the trace quantities generated by the software results. The increment or decrement in the expression must be interpreted in the folds.

The graphical representation is generated using the Kyplot Software in the form of bar charts. The bar charts clearly represent the effects of the toxicant and the plant drug with reference to the control expression with standard errors. The damage has been portrayed significantly in the expression of the MBC treated animals and the gene expression of the animals administrated with the plant drug are expressing that the damage is being cured by the plant drug (Figs. 2 & 3). The difference in the dosage level is also having its impact on the results. The microtubules got damaged because of carbendazim. It is a microtubule poison by disrupting them (Nakai et al., 1995). As they were damaged, the expression is less in the MBC treated animals when expressed with tubulin. Whereas the positive do not have such damage and the animals which were getting the plant drug were giving higher trace quantity than the MBC treated animals' expression but relatively less than the control expression.

On the other hand, treatment of *C. orchioides* prevented all these pathological manifestations in the testis and the abnormalities associated with sperm. The sperm and the testes are confirming the restoration of nearly normal characteristics as they are comparable with the control. The microtubular damage in the animals treated with the MBC alone is clearly depicted in the RT-PCR results. The curative property of the *C. orchioides* is confirmed by the results of the gene expression. The poisoning effect of the carbendazim is nullified by the treatment of the *C. orchioides* (Figs. 2 and 3).

The RT-PCR results conforms the guesses made in the past studies. It has been proved that the microtubules occupy the middle piece in greater extent. Davidse and Flach (1977) proved that the fungicidal property of carbendazim resides in its ability to bind to microtubules, thereby inhibiting mitosis and meiosis. In addition, these chemicals prevent polymerization of tubulin and thereby, prevent the formation of fresh microtubules (Dustin, 1984). Thus, it can be conformed that the plant drug has the efficiency of curing reproductive toxicity and the dosage level can be determined by depending on the body weight of the organism. As the results of the RT-PCR are confirming the efficiency of the *C. orchioides*, they can be used as the curative for the toxicity.

## Discussion

Carbendazim is a well known fungicide to cause the reproductive toxicity by accessing mainly the Sertoli cells and the lumen of the testis, the microtubules and the cytoskeletal elements and detoriating them by its fungicidal property. It exhibits its fungicidal action through their ability to disrupt microtubule formation (Akbarsha et al., 2000). It binds to the β-tubulin subunits of microtubules, thereby inhibiting mitosis (Davidse and Flach, 1977; Burland and gull, 1984).

On considering the β tubulin, as a result of depolymerization of the existing microtubules and prevention of the formation of fresh microtubules, the metaphasic chromosomes fail to separate and hence, cell division is arrested. Depolymerization of the microtubules would most likely begin at their plus ends and progress toward their minus ends which are directed toward the seminiferous tubule lumen.

Regardless of the mechanism, the disruption of microtubules can be attributed to the inhibitory action of carbendazim on microtubule formation (De Brabander et al., 1976). MBC binds to tubulin subunits results in the dramatic decrease or disappearance of microtubules in the Sertoli cells (Russell et al., 1981a, b). The β-tubulin and β actin are specific genes for the microtubules and cytoskeletal elements, the damages are clearly depicted in the expression and its quantification. Moreover, recent studies indicated that the testis-specific effects of MBC may arise from inhibition of microtubule-dependent processes unique to the testis. (El-Sabeawy and Miller, 1995)

The damage caused by the fungicide by the depolymerization activity resulted in the detoriation of the entire mechanism of the spermatogenesis. The spermatogenic process was impeded by causing necrosis of cells in division, both mitotic and meiotic, and preventing pachytene spermatocytes from completing the second meiotic division (Hess and Nakai, 2000). The microtubule damage is clearly depicted by the lower quantification value of the MBC treated rats for β actin and β tubulin than the control and cured rats. The fungicide is known as the microtubule poison and it affects the cells during the cell division, thereby preventing meiosis. The entire spermiogenesis cycle is getting disrupted by the necrosis in the mitotic and meiotic cells in the testis (Nakai and Hess, 1977). Effects seen on cells in meiosis peaked at 12 hours. Necrosis occurred most frequently in metaphase primary and secondary spermatocytes (Hess and Nakai, 2000).

The present study provides evidence for disruption of cytoskeletal elements other than microtubules by MBC, as envisaged by Nakai and Hess, 1994. The abnormalities in the cytoskeletal structure may be sought to the disruption or detoriation of actin and its element by MBC. The damage is clearly seen in the gene expression with low band intensity and the low value of the trace quantity when quantified using the quantifying software. The poisoning effect of carbendazim is well-expressed in all the genes used for study. While dealing with the rats administrated with the plant drug produced the results which as similar as the control. They are relatively higher than the MBC treated rats in their quantification values which prove that they are cured and the abnormalities are nullified by the effect of the treatment. Thus, it can be conformed that the plant drug has the efficiency of curing reproductive toxicity and the dosage level can be determined by depending on the body weight of the organism. As the results of the RT-PCR are confirming the efficiency of the *C. orchioides*, they can be used as the curative for the toxicity.

**Fig. 2: β Actin expression analysis of control and MBC treated rats. L-Marker; L-control; L2-MBC treated; L3-Pland drug 100mg; L4-Pland drug 200mg.**

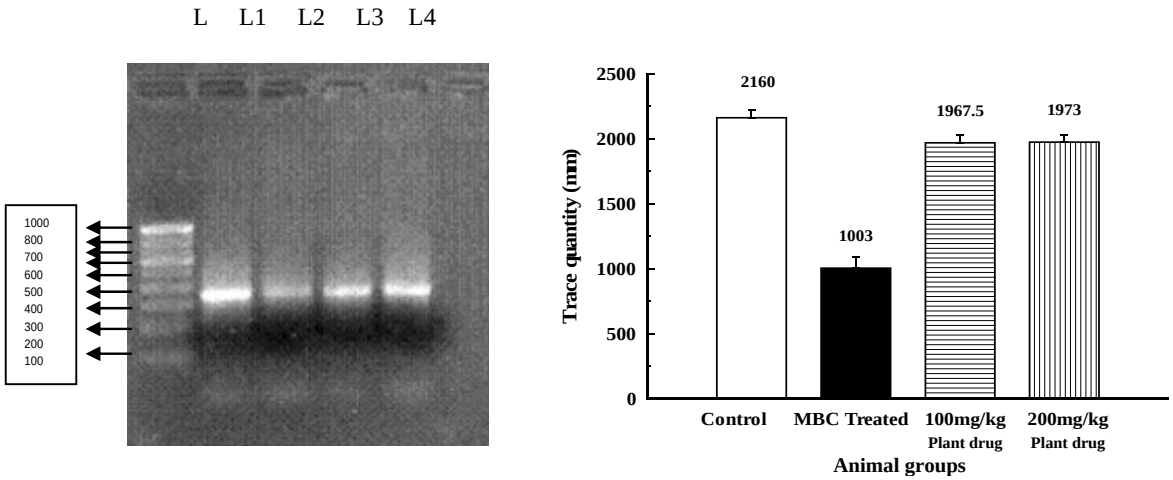
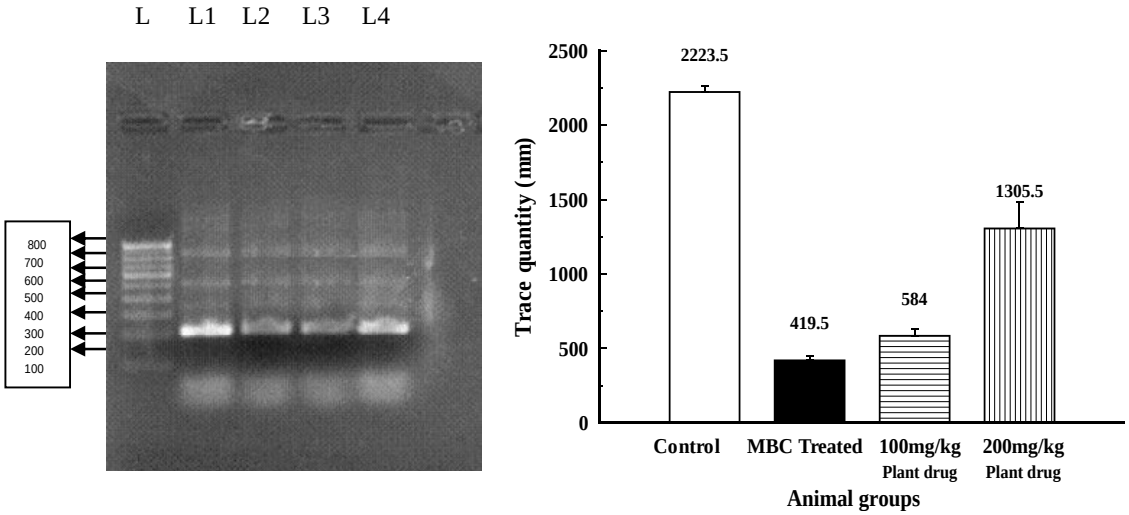


**Fig. 3: β-Tubulin expression analysis of control and MBC treated rats. L-Marker; L-control; L2-MBC treated; L3-Pland drug 100mg; L4-Pland drug 200mg.**

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