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ANTIDIABETIC AND ANTIOXIDANT POTENTIAL OF *COPRINUS COMATUS* IN ALLOXAN-INDUCED DIABETIC RATS MODELS

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Abstract

Background: Mushrooms are used as nutraceuticals from a long time. In the present era they got attention of researchers due to a large number of bioactive compounds. *Coprinus comatus* (*C. comatus*) is a therapeutic mushroom helpful in the management of Diabetes Mellitus. The pancreas having beta cells were very susceptible to Reactive Oxygen Species (ROS) attack, producing an unbalance insulin productions.

Methodology: The study was organized to investigate the *C.comatus* methanolic extracts for antidiabetic and antioxidant activity. Sixty (60) male rats were divided into five groups, G1 was consist of normal control group (NC), G2 was positive control (PC) G3 was Diabetic Control (DC) (G4 and G5: Giving 200 and 400 mg/kg BW mushroom extracts). All the parameters were analyzed using the ANOVA software.

Results: On the bases of obtained results, the 400mg/ kg BW *C. comatus* extracts significantly reduced the fasting blood glucose level (FBC) up to 22.86% in G5 group.

Keywords: Mushrooms, Bioactive Compounds, Antioxidant and anti-diabetic Activity

INTRODUCTION:

Diabetes mellitus (DM) which is a metabolic disorder mainly characterized by the hyperglycemic conditions due to the low secretion of insulin by pancreas. The peoples suffering from diabetes mellitus in the hyperglycemic states, causes the productions of free radicals. Moreover, these free radical demolished by the antioxidants. In DM, the hyperglycemic diabetic patients, having an experience to decrease their enzymatic anti oxidents. The enzyme, superoxide dismutase (SOD) play a key role to defend and protect β-cells from the dangerous effect of (ROS) and free radical attack.

The exogenous antioxidants are quite necessary, for the lack of these enzymatic antioxidants, containing *C. comatus* mushroom. The mycochemical compounds founds in *C.comatus* are quercetin and rutin flavonoids. The compounds are helping in preventing the numerous complications of DM and neutralizing of free radicals. The reactive oxygen species (ROS) inhibitions by the quercetine and rutin flavonoides are ideal bioactive compounds which provides and helpful in neutralizing the free radical and are more effective against the cardiovascular and diabetic complications.[4] The flavonoides having a strong antioxidant potentials, where it protects the bodies against the damages caused by the reactive oxygen species by giving the hydrogen atoms and binds with free radical to form stable flavonoides radicals. As a result, it can inhibits the lipid peroxidations damages of the cells and prevent the complications like diabetes mellitus.[5]

The (WHO) has approved and recommended the mushrooms as a herbal medicines. But research bioassay test as a inhibitors of the DPP-4is still insufficient. It need to be carried and considered that DPP-4 play a key role within the intestinal system.[7] Moreover, methanolic extract of fruiting body of *C.comatus* and alloxan inductions to create the hyperglycemic rat permanentlyis yet exceptional.On the bases of these, intense need is required to explore the activity of fruiting bodies methanolic extract of *C.comatus* on rats induced alloxan. The aims of this studies to calculate the changes in DPP-4 enzymes as a antioxidant to analyse and determined the dose of *C.comatus* extracts, which are more affective in decreasing the blood glucose level.

MATERIALS AND METHODS

The fruiting bodies of *C.comatus* mushroom were obtained our surrounding localities of District, Bannu having a No of CC, 1974 HU, Mensehra kpk Pakistan. The Rats were obtained from National Institute of Health (NIH) Islamabad, Pakistan, and Alloxan (Himedia), Glucometer and Rats DPP-4 Elisa kit Cat. No. L1986Mc[8] were used.

Experimental design and treatment of the animal

A complete random model and test treatment were carried out with a control group. A simple randomly sample procedure were used, where, 60 rats with a 170-200 BW were brought and divide into five groups and each group consists of 4 rats. The rats were placed in a perforations steel cage and kept in a climatic control room. The rats were giving feed with a grinding grain twice a day and water for drinking. The cages were being kept clean in every day, and under the base was removed to protect the rats from the disease .The five groups are G1:Normal Control (no treatments; only water and food), G2: NC (Alloxan induced; negative control), PC (gulcophage 40mg/kg BW; Positive control) G1(200mg/kg BW of Extract), G2(400 mg/kg body weight of extracts).The extracts of *C. comatus* and the gulcophage were start on day 5 by the alloxan induction to rats for 21 days.

Mycochemical Screening

The flavonoides test was carried out by giving a heat to 2mL amount of *C.comatus* extracts for 5-10 minutes. By mixing of 0.12 mL of HCL shows a yellow, Orange, and red color. The alkaloids and terpenoides test was also conducted by the addition of drag and orff-boucherd reagent and three drops of HCL before by the additions of 2 mL H_2SO_4 into a *C.comatus* extract.

The saponine were also recognized and tested in hot water contains the extracts with 2-3 mL methanolic solution. The extracts were cool down and shacked for 12- 28 seconds.

Crude extract methodology of *C.comatus*

The fruiting body of *C.comatus* were into a small slices and then dehydrated at 40-5°C and then grinded into powder form, A 200 g of a sample powder is mixed in a beaker with 1:5methanolic solvent , and placed and allowed for 24 hours , after which filtration was carried out with a filter paper. The bulky extracts obtained by the process of loosing of water is weighed and is ready for further process.

Alloxan induction

The experimental rats were placed in a controlled climatic room for 6-112 days. The temperature of the room was kept constant between 15-30°C with 55-60% moisture contents. Consequently, 0.5mL of Alloxan was giving intravenously at 40 mg/kg body weight dose in buffer citrate solution 0.1M at pH 4.5. All the animals were found in a sound and good health condition before the experimental process.

Collections of the blood serum and measurement of blood glucose

For the measurement of main parameters, a blood serum was collected, but before the experimental process, all the animals were placed in fasted conditions for 11 hours. After alloxan inductions, the 1st blood glucose were taken from the internal vein on day 4th. The taking blood was placed on glucose strips. On 21th day, after giving the *C.comatus* extracts, a blood sample was obtained from the lateral veins of the rats, and then calculated and measured the final blood glucose level by using the glucose strips, and get plasma by using a centrifuged machine at 6000 rotation per minutes, and placed in a cool refrigerators with a temperature of 2-10°C.

Animals recommendations

The committee recommended and give the approval of the process of Health research ethics, treatment of animals and experimental research design, faculty of biological sciences, University of Science and Technology Bannu, KPK Pakistan. The main requirements of this animal approval committee are based on the principals of reductions, replacement and refinement. Moreover, it is considered the principals of pain, injury and diseases, thirst and hunger and the appearance of their natural behavior of the rats.

Statistical analysis

All the results were indicated as mean ± standard error of the mean (SEM). All the experiments were brought in triplicates. Graph Pad Prism 5 was run for data analyses and management.

RESULTS

The experimental research showed the process of extractions of 200g of *C.comatus* fruiting body by using 1000mL methanolic solvents yields 2.20g of sample extracts. The table 1 indicates the mycochemical compounds of the mushroom of *C.comatus* extracts used methanolic solvents.

Table (1): Classificationsof *C. comatus*mycochemicals compounds.

No	Mycochemicals Compounds	Chemicals Reagent	Results
1	Flavonoids	Mg/Zn + HCl +Amyl Alcohol	Reddish orange (++)
2	Alkaloids	Mayer, Dragendorff, dye	Dark brown (+)
3	Terpenoids	HCl + H_2SO_4	Purplish black (+)
4	Saponin	Distilled water + boiling + methanol	Formed Foam (++)

Note: The plus signs in table were indicated the following informations: + (adequate concentration), ++ (moderate concentration), and +++ (strong concentration).

The methanolic extract of *C.comatus* mushroom contains the mycochemicals compounds such as flavonoids, alkaloids, terpenoids, and saponins. From the (Table 1) it indicates that flavonoides showed a moderate compounds concentrations with a indicators of a color changes to reddish purple. By the addition of an adequate Mayer, Dragendroff and Bouchardat dye, the alkaloids compounds showed a color changes to darkbrown. Similarly the color change to blackish purple also indicates the presence of an adequate amounts of terpenoides compounds. A reasonable concentrations of saponine was also identified, by the addition of 3 drops of methanol in *C.comatus* extract, heated and shake thouroughly for 1 minutes and reasonable foam was formed.

From the previous it has been identified that the fruiting body of *C.comatus* methanolic extracts contains a reasonable amounts of flavonoids compounds, low level of alkaloid and saponine.[7]In other studies, by using the ethyl solvents extracts of sample with a concentrations of 100(ppm) and was identified to contains a strong concentrations of flavonoides and terpenoides.[2] Similarly, the similar results were also been expressed from the methanolic extract of mushroom *C.comatus* fruit in body, consists of reasonable and small amount of flavonoides and saponine contents.[5] In other studies, 71% and 94% ethanolic *C. comatus* extract was investigated to contains phenolics compound and acts as an antioxidant in DPPH radicals.[9]

The flavonoides and polyphenoles are the major components acts as a antioxidents, where's saponine and alkaloids acts as a antidiabetic agents. Moreover, the compounds alkaloid acts as a inhibitor of the enzyme DPP-4 thathumiliate the GLP-1 harmone[10]. From the previous knowledge, it had showed that alkaloids has a inhibitory activity α-amylase and α-glucosidase and DPP-4 enzyme.

They play and act as an inhibitory functions in a highly developed glycations ending products (AGEs), releaseness of insulin from the beta cell of pancrease and their sensations, and improvements of the glucose uptake[11]. Furthermore, saponins also play a great role in inhibitions of uptaking of the excessive glucose in the intestinal mucosa to decrease the catabolic activity by raising the adenosine monophosphate kinase assay[7]. The other role is to increase the insulin level to maintain the permanent production and minimize the glucose level in blood. From the previous research studies, it has been investigated that saponine diminish and decrease the complications produce due to fatness and inflammations and prevent liver complications and hurtness in insulin harmone resistance rat.[12] The diabetic rats was also treated by the *C.comatus* extracts, the active compounds founds them decrease the glucose levels in blood.

From our research, it had been shown, that the *C.comatus* fruiting body bulky extracts contains the quinic acid. The quinic acid activity as a antioxidant compared to the extract *C.comatus*fruting bodies were largerwith 3μmol/L inhibitions.[13] Two flavonoids compound consists of high amount of quinic acid 50 mg/100g and the quercetin compound at 3.10mg/100g.[14] The bioassay as a antioxidant is quite necessary to give protection to pancreatic β-cell from the attack of free radical. Other study stats that methanolic extract of *C.comatus* cap give off the free radical DPPH with an EC_{50} value of 0.870 mg/mL, againsthydrooxal (OH^-) radicals 3.224 mg/mL, and against O^{2-} radicals 24.41 mg/mL.[15] So, the *C. comatus* bulky extracts play a key role in neutralizing the OH^- radicals.[16]

From the measurement of the blood glucose level, it has been showed that after 95 hours induction of the alloxan, the fasting blood glucose level was increased upto 150 mg/dL. Likewise, the diabetogenic agents also increase the fasting blood glucose level > 150mg/dL and the highest was recorded upto>186mg/dL (Figure1).

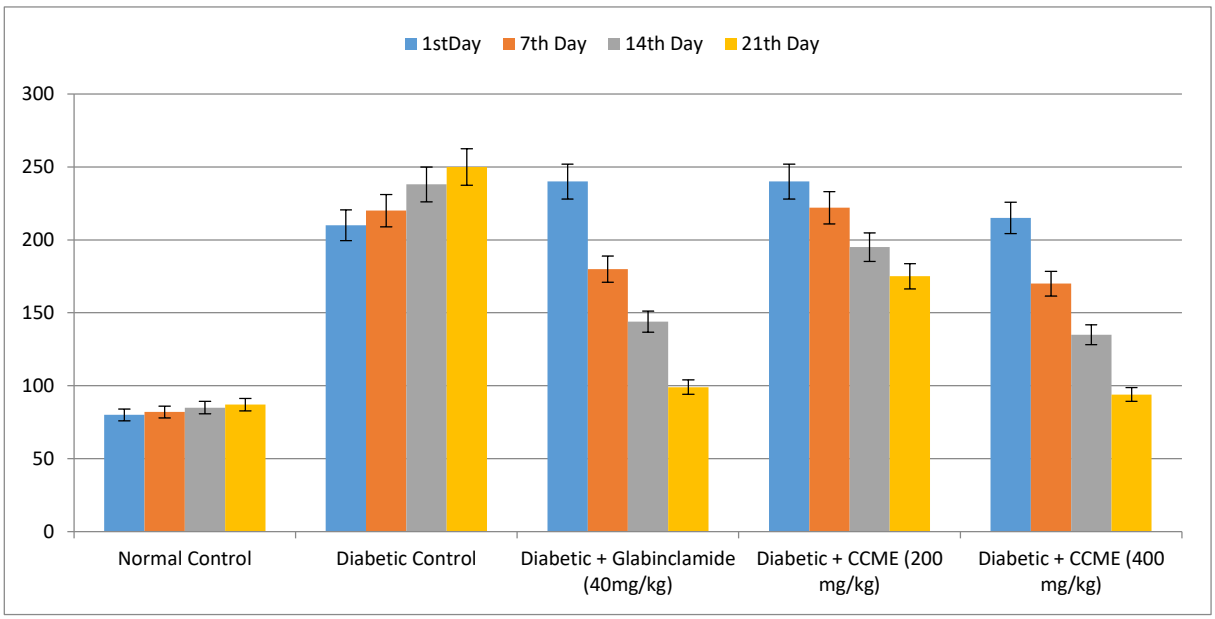

Figure. 4. 15: Graphical representation of mehtanolic extract of *Coprinuscomatus* on blood glucose level of normal and diabetic rats in alloxan induced diabetic rats.

The greatest and the low level of blood glucose was recorded on 7 day, G1 and healthy group, with a 189.4 mg/dL and 99.01 mg/dL respectively. The highest blood glucose level was minimized on 21 day after giving the dose of *C.comatus* methanolic extracts and glucofage. The administered dose significantly minimized the glucose level in blood of the group ($p < 0.05$) (Figure 1) in the G1 group by 14.45%.Similarly, the NC group has remarkable increase in fasting blood glucose level shown in (Fig 1) from 177.5mg/dL to 188.5 mg/dL and 248.11 mg/dL on 21 day respectively.
The percentage decreasing was calculated by the comparison of early levels with the values get at the time of measurement. The positive control groups indicates decreasinglevel on day 21 and 21 from 166.5mg/dL (Day 7), 145.7mg/dL (Day14), and 139.8mg/dL (day 21) respectively. On day 21 the G1 group showed a remarkable decrease ($p< 0.05$) with a 25.88% (188.5mg/dL to 137mg/dL), in the G2 group 19.50% (169.5mg/dL to 133.4mg/dL) and G3 group 23.4% (187,31mg/dL to 139.1mg/dL) (fig.1).

This results indicates that *C.comatus* extract showed a remarkable decrease blood glucose level for 21 days (Fig 1). In the meantime, the negative group, the level of the blood glucose raised on day 14th and 21th, while the greater minimization was investigated in group G1. On day 14th and 21th day, it is minimized to 14.65% and 25.76% as compared to the 1st day. It is clear from the study, that *C.comatus*bulky extracts can minimize the fasting blood glucose level by an average of 22.93% (Fig 1).

It is clear from the study, that *C. comatus* bulky extracts can reduce the fasting blood glucose level by an average of 22.86% (Figure 1).
From the earlier studies it had been clearly indicated that methanolic extracts of *C.comatus* at 200 mg can minimize the blood glucose level upto 22.38%.[7]Additionally, the dose administerly at 400 mg can help greatly minimize blood glucose level upto 27.70%.[5]
The other studies showed that induction of alloxan at 200mg at the dose of 400 mg extract of *C.comatus* showed a 49.08% decrease after 21 day, whereas the rats, lacking the treatment of extracts indicated an increase of 48.90%.[17]

The alloxan directly affects the pancreatic β-cell by inflowing by the glucose transporter (GLUT)-2 means. It stimulates the formations of nitric oxide (NO-) and causes DNA alkylation.[18] The uptaking of alloxan in pancrease beta cell by GLUT-2 receptors causes the oxidative stress reactions due to the

creation of $NO^-$and reactive oxygen species (ROS) productions causes inflammations which diminishes the pancrease beta cell. The consequent affects are the oxidative stress, cell membrane lipid perioxidations, and less amount of the ATP formations. The damages of the pancreatic β-cell, can lead to the less amount of the insulin productions and lead to increase the fasting blood glucose level.[19] Such type of the conditions where alloxan cause cell necrosis, cell memebrane damages and increase the blood pressure change accordingly their physiology, biochemistry and immune power.
The free radicals are produces such as hydroxyl radicals and hydrogen peroxide are the increased glycoxidations. This can cause the formations of AGEs, which produce many free radicals and can damages of the pancrease beta cell. The formations of crossing link b/w AGEs molecules with their receptors can lead to the enlargement of the diabetic diseases.[20] Moreover, the flavonoid contents in *C.comatus* extract can repress the reactive oxygen species formations and protects the development of pancrease beta cell damages. Glutathione peroxidase levels were observed in diabetic rats in 21 day by using the methanolic extract of *C.comatus* and gulcophage administrations (Table 2).

Table (2): GPx level in alloxan-induced hyperglycemic rat.

No	Treatment groups	GPx levels (U/mL)	% Increase
1	HC(NO treatment)	22.1 ± 4.83 [ab]	159.6
2	NC (40 mg/kg BW)	7.72 ± 4.32 [a]	-60.64
3	PC (40 mg/kg BW)	33.0 ± 16.73[b]	274.41
4	G1 (extract of 200 mg)	38.0 ± 3.34 [b]	340.16
5	G2 (extract of 400 mg)	37.4 ± 15.40 [b]	334.41

"Notes: Data is presented as mean±standard deviation (n=4). The various small alphabets shows the variation in significance where ($p \leq 0.05$)".

The *C. comatus* methanolicextracts at 200mg and 400mggiven doses to diabetic rats remarkably increase the antioxidant level of GPx ($p < 0.05$). The groups which get the methanolic extracts with a dose of 200 and 400mg has a GPx bioassay of 40.24 U/mL and 37.4 U/mL correspondingly, which was greater as compared to the normal control group. In the meantime, theGPx level in the normal control group were quite under than the normal limit of 7.72U/mL.The normal GPx level in the tested animals 18-24 U/mL,[5] where the *C.comatus* normally consists the flavonoids compounds. From the earlier research, it has been indicated that the given dose of *C.comatus*methanolic extract 200mg and 400mg increase the enzymatic antioxidant of GPx level of 38 U/mL and 37.4U/mL.[2]

The earlier studied indicates that the flavonoide compounds can stop the lipidperioxidation by giving the hydrogen ions (H^+) and minimize the tissue damages by free radical.[2] Moreover, the *C.comatus* consists of the ergothionine compounds, just like a methionine. On the other hand, the GSH substrates restore with an ergothionine and GPx assay return to normal.[17]

The *C.comatus* containing the flavonoides contents like rutin and tocopherols are capable for the breakdown of the lipid peroxidations chain reactions. More OH- flavonoides can interact free radical by giving H+ ions and minimize their assay and alter the radical molecules into a flavonoides radical which is not reactive against the cell.[23] The earlier research studies indicats that the contents in methanolic extracts were 17.39 mg/L or about 33.9%, which are very cost effective of *C.comatus* in suppress the free radical. So, as a result the insulin remainsmost favorable and the level of blood glucose were kept up in tested animal which was induced by alloxan. The extracts of *C.comatus* was also containing an iron (Fe) minerals of 72mg/Kg. The catalase bioassay can minimize the peroxide components in cell, such as H_2O_2, and can change them into H_2O and O_2. Additionally, it consists of the cofactors which, help the biosyntheses of Cu-SOD, Mg-SOD, and Zn-SOD, due to the minor elements contents of Cu 11.16 mg/Kg, Mg 1335 mg/Kg, and Zn 32.74 mg/Kg which, permit the process indirectly to defend the cell from the oxidative stress.[24]
After 21 days, the dipeptidyl peptidase-4 level was investigated by using the methanolic extract of *C.comatus* and gulcophage administrations.

Table (3): DPP-4 levels in Aloxane-induced hyperglycemic rat model.

S.No	Treatment group	DPP4 level (U/mL)	% Decreasing of DPP-4 as Compared to NC Group
1	G1. (NO treatment)	77.34± 2.7[a]	53.58
2	G2. NC (40 mg/kg BW)	177.44± 3.6[c]	+126.19
3	G3.PC (40 mg/kg BW)	95.22± 4.6[b]	46.68
4	G4 (extract of *C. comatus* 200 mg)	97.37± 5.5[b]	45.70
5	G5 (extract of *C. comatus* 400 mg)	82.26± 4.3[a]	51.75

"Notes: Data is presented as mean± standard deviation (n=4). The various small alphabets shows the variation in significance where ($p \leq 0.05$)".

Table 3 indicates significant variations of DPP-4 after and before the treatments of methanolic extracts of *C.comatus*. Moreover, 200mg and 400mg doses were contrasted to the PC and NC groups of rats. The high level of DPP-4 enzyme was noted in negative control group with a 177.44 U/mL, and the lower level wasrecorded in the healthy control group with a 77.34U/mL. Generally, the proper management of *C.comatus* extracts were usually below as compared to the normal control group. At the dose of 200 mg methanolic extracts indicates the DPP-4 level at 96.37U/mL, whereas at 400 mg/mL extract showed a decrease level of DPP-4 at 82.26U/mL. The percent reduction in DPP-4 enzyme level as contrasted to the normal control group indicates that group (G2) has a highest decrease with a 52.33%.

Accordingly, the result showed, that the level of DPP-4 hormone in the diabetic rats groups reduced. From the ANOVA investigation, it is clearly indicated that the results were remarkable at $p \leq 0.05$. A significant reduction in the level of DPP-4 enzyme in the diabetic treatment group was possible. Comatin will generally stick up with the DPP-4 enzyme to stop the DPP-4 enzymatic activity.[5]

The previous study shows that comatin acts as a DPP-4 enzymeinhibitors and interacts with a GLP-1 hormones. It replace the DPP-4, where it connects with GLP-4 harmone active sites. Since the DPP-4 enzyme can no further connects to the GLP-1 harmone, and the deactivations of the DPP-4 are reduced. Moreover, the comatin cannot effects the GLP-1 and their insulinotropic assay can generally active.[6]

The rutin founding in *C.comatus* can develop their antioxidant defence in pancreatic cells and protects the cells from losses due to the oxidative stress.Therutin as an antioxidant activity is very important to defend the pancreatic cell from damages.[7] An earlier studies indicates that the decreasing of DPP-4 enzyme communicates with the rising levels of GLP-1 hormone after the oral treatments of *C. comatus* extracts. The 400mg methanolic extract of *C.comatus* demonstrates the highest prominent effects of minimizing the DPP-4 enzyme level in hyperglycemic rats model induced with alloxan 40mg.[5]

The DPP-4 enzyme play key role, due to its increasing, it effects directly the incretin harmon. The earlier studies have indicated that DPP-4 inhibitions can minimize the glycosylated haemoglobin, effecting the functions of pancreas beta cell in insulin production blood pressure.[25] So, the role of *C.comatus* bioactive compounds which contains the flavonoides as a antioxidents and the effects on rising GPx can give the pancrease beta cell defend from the free radical. The insulin syntheses and secretions will generally run to continue their inhibitory effects of *C. comatus* against the DPP-4, which are concerned with increasing of the insulinotropic bioassay.[7]

CONCLUSION

These results clearly indicated that the *C.comatus* methanolic extracts in hyperglycemic rats induced by the alloxan at various doses can lessen the values of GPx and DPP-4 in hyperglicemic rats. Moreover, the extracts at 400mg/kg body weight affectively minimize the fasting blood glucose level and DPP-4 level, whereas raising the GPx enzymatic antioxidant.

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Authors have declared that no competing interests exists

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