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***In Vitro* Analysis of Anti-Diabetic Activity in *Catharanthus roseus* Root Extract with Piperine as a Bio-Enhancer**

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Abstract: *Catharanthus roseus* is a traditional plant which is more common in subtropical gardens through the year. The present study evaluated the anti-diabetic activity of the extract of *Catharanthus roseus* roots with Piperine as a bio-enhancer. Alarming rise in the incidence of Diabetes mellitus has become a major concern around the globe. Various studies have been conducted by the researchers in the past on the anti-diabetic activity of *Catharanthus roseus*. But the obtained result was not up to par. So we opted for a bio-enhancer. *In vitro* test analysis has been carried out in this study. The phytochemical analysis was performed to check the presence of major alkaloids in the methanolic crude extract of *Catharanthus roseus* and Chloroformic crude extract of *Piper nigrum*. α -amylase inhibition assay was conducted to reveal the anti-diabetic activity of the crude extracts of *Catharanthus roseus*, *Piper nigrum* and the combined extract. α -amylase inhibition assay was conducted to analyse the anti-diabetic activity. In the combined crude extract, DPPH assay and Fe^{3+} reducing power assay were performed to analyse the anti-oxidant property. Anti-bacterial assay was also performed in the combination crude extract using Agar well diffusion method. The combination crude extract is a potential source of anti-inflammatory agents. Thin Layer Chromatography is a separation technique which confirmed the presence of the required phytochemicals in the crude extract. Thus, we concluded that the anti-diabetic activity of *Catharanthus roseus* could be enhanced with Piperine to a greater extent.

Keywords: α -amylase inhibition assay, *Catharanthus roseus*, Fe^{3+} reducing power assay, *Piper nigrum*, Thin Layer Chromatography, DPPH assay

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Introduction

Diabetes mellitus commonly referred to as diabetes, is a metabolic disorder in which there is a prolonged high blood sugar level. Over 425 million people across the globe are affected with

Diabetes mellitus. Being the diabetes capital, India has around 62 million suffering from this disorder. This represents 8.8% of the total adult population of the country, equally among both women and

men (Shingate *et al.*, 2013; Imad *et al.*, 2014.). Trend suggests that rates will continue to rise at an exponential rate. Medicinal plants have been in use since ancient times for the control and treatment of Diabetes mellitus. *Catharanthus roseus*, commonly known as the Madagascar periwinkle is a species of flowering plant in the family Apocynaceae (Ayodhya *et al.*, 2010; Waleed *et al.*, 2015; Prakash *et al.*, 2018). It is an evergreen herbaceous plant growing 1 m tall. *Catharanthus roseus* is found to be a rich source of alkaloids like Vinblastine, Vincristine, Vindoline etc. (Verma *et al.*, 2007; Chandrasekaran *et al.*, 2014). Many studies had proven the anti-bacterial, anti-inflammatory and anti-oxidant properties. Studies were also conducted to prove the anti-diabetic (Sikarwar *et al.*, 2012) activity exhibited by various parts which is contributed by the major alkaloids (Prakash *et al.*, 2018). Piperine is the major alkaloid found in the flowering vine, *Piper nigrum* of Piperaceae family (Ranveer and Siddharaj, 2012). Piperine is an effective bio-enhancer through various research works.

The antidiabetic activity of extract is found comparable to other components such as the Glibenclamide. Thus, leaves of other plants can also use as anti-diabetic drug (Lel *et al.*, 2011; Amin *et al.*, 2021) even though it is difficult to extract a large quantity from an easier technique. Dietary supplements can also improve the blood glucose level, eventually it can produce some of the other factors also to improve such as body weight (Ayodhya *et al.*, 2010). The present study aimed to evaluate the anti-diabetic activity of the crude root extract from *Catharanthus roseus* and Piperine will be used as a bio-enhancer to enhance the reported anti-diabetic activity of the root extract. In various other studies lipid profile has been checked with their results showing a drastic change in the blood glucose level (Mohammed Hamdoon *et al.*, 2013). The results from this study are analyzed by applying the extract to pancreatic cell lines with the control from various previous studies (Kratika and Sharmita, 2011).

Materials and Methods

Collection of plant materials:

Fresh plants of *Catharanthus roseus* were collected locally. The roots were coarsely ground to a powder. The clean and fresh fruits of *Piper nigrum* were collected from the authorized horticulture. The collected fruits were dried and finely ground to a powder (Shingate *et al.*, 2013).

Crude Extraction:

Catharanthus roseus root:

10 g of the powder of *Catharanthus roseus* root was mixed with 100 ml methanol. This mixture is left undisturbed for 3 days. This procedure is carried out in order to break down the cell wall of the components in the mixture. Thus, the phytochemicals would be released into the solvent. This makes the phytochemical analysis more accurate as the phytochemicals are let free in the solvent (Prakash *et al.*, 2018).

Piperine extraction:

The fruits of *Piper nigrum* were dried under sun. These fruits were then finely ground into a powder using a mixer grinder. 50 g of the powder is then mixed with 100 ml of Chloroform. This is left undisturbed for a day. The Piperine extraction was carried out by Dichloromethane method. 10 g of ground pepper powder was refluxed with 20 ml of Dichloromethane for 20 min in a round bottom flask. Condenser was attached and water was allowed to run through to condense the dichloromethane vapours. Later on, the flask was cooled and filtered through Buchner funnel. The extract was then treated with acetone and hexane.

Qualitative Analysis of Phytochemicals:

Crude extract of *Catharanthus roseus* and *Piper nigrum* were subjected to phytochemical screening.

Test for Alkaloids:

To a 2 ml of crude extract (free from ethanol) 10 drops of con. HCl and 10 drops of Picric acid was added. To the resulting mixture 10 drops of Mayer's reagent was added along with 10 drops of

concentrated Hydrochloric acid. Formation of yellow colour indicated the presence of alkaloids (Kabesh *et al.*, 2015). A sample test tube of the filtrate was reserved for reference. (Vikash and Srivastav, 2021).

Test for Phenol:

To a clean test tube 2 ml of crude extract was added with few drops of ferric chloride solution. The colour of the solution was turned to a bluish black colour which confirmed the presence of phenolic compound (Kabesh *et al.*, 2015; Vikash and Srivastav, 2021).

Test for Tannins:

To 1 ml of crude extract added 1 ml of FeCl₃, Methanol (MeOH) and a few drops of lead acetate solution. The presence of blue black colour indicated the presence of Tannin (Kabesh *et al.*, 2015; Vikash and Srivastav, 2021).

Test for Terpenoids:

2 ml of extract is initially taken in a clean test tube to which 0.5 ml of concentrate H₂SO₄ was added along with 0.5 ml of chloroform. The presence of brown colour indicated the presence of Terpenoids (Kabesh *et al.*, 2015; Amin Mir *et al.*, 2018; Vikash and Srivastav, 2021).

Test for Glycoside:

Glycosides were tested by dissolving 5 ml of extract in 3 ml of acetic acid and carefully added 1 drop of concentrate H₂SO₄, a reddish brown colour was formed indicating the presence of Glycosides (Hester Hodges, 2017).

Test for Flavanoids:

To 1 ml of extract added 1% NaOH and mixed it well which formed a yellow colour confirming the presence of flavonoids (Kabesh *et al.*, 2015; Vikash and Srivastav, 2021).

Test for Saponin:

1 ml of extract was diluted with 5 ml of distilled water and shaken well. The solution formed a precipitate of 1 cm height which was absent in the solution which may be due to the adding of *Piper*

nigrum. This indicated the absence (Kabesh *et al.*, 2015; Vikash and Srivastav, 2021).

Test for Steroids:

1 ml of the extract was mixed to 1 ml of chloroform and concentrate H₂SO₄ sidewise which was added to acetic anhydride. A red colour change was noted which indicated the presence of steroids (Kabesh *et al.*, 2015).

Thin Layer Chromatography:

The extract was added as a small spot on one end of the thin layer plate (silica gel GF24 plates 20×10cm) above 1 cm. Plate was allowed to dry. Then, it was placed in a beaker containing toluene and chloroform in 1:1 ratio. The samples were now run towards the other end of the plate. Approximately, $\frac{3}{4}$ th of the plate was ran with the sample. The plate was visualised under the UV light and colour spots were absorbed.

Quantitative Analysis:

Phenol Estimation:

Total phenolic content of the extracts were determined by using gallic acid as the standard and the calibration curve was constructed by the absorbance value of standard gallic acid. 1 ml of the extract was taken in a clean test tube and added 1 ml of Folin-Ciocalteu reagent to it. Now, mixed it with 20% of Sodium carbonate (Vikash and Srivastav, 2021). The phenolic compounds, Salicylic, Ferulic, Gallic and Chlorogenic acids also known as phenolic acids are particularly important for the phenolic concentration estimation of any plant extract (Ane Patricia *et al.*, 2020). The extract was incubated for 45 min at 40 °C. The absorbance value was measured at 765 nm by spectrophotometer (Kabesh *et al.*, 2015).

Flavonoid Estimation:

1 ml of extract was taken in a clean test tube. Then, 500µL of 5% Sodium nitrate was added to it. Also, added 500 µl of 10% aluminium chloride (Vikash and Srivastav, 2021). Then, 50 µl of 1M NaOH was added to it. It was incubated at room temperature for 10 min. The absorbance value

was measured at 510 nm by the spectrophotometer (Kabesh K *et al.*, 2015; Ane Patricia *et al.*, 2020).

α – amylase inhibition assay:

Acrabose is an alpha glucosidase inhibitor which decreases intestinal absorption of carbohydrates to manage the type 2 diabetes (Ane Patricia *et al.*, 2020). The extract (50-300 µl) was taken in a clean test tube and made up to 1 ml using D.H₂O or anti-diabetic buffer. 10 µl of α-amylase was added to this. This was then incubated for 10 min. Then, added 500 µl of starch. Then incubated it for 1 h. Added 100 µl of HCl and 200 µl of Iodine. The Optical density reading was taken by spectrophotometer at 565 nm (Sathya *et al.*, 2008; Ganiyu *et al.*, 2013).

Fe³⁺ reducing power assay:

Various concentration of the extract (20-120 µg/ml) was taken in clean test tubes. Those were then made up with 1 ml of MeOH in each test tube. 1 ml of phosphate buffer of pH 6.6 was added to each test tube and incubated for 30 min at 50°C. Then added 300 µl of 10% Trichloroacetic acid (TCA) and freshly prepared 0.1% ferric chloride (Jayanthi and Lalitha, 2011; Ganiyu *et al.*, 2013). The absorbance was read at 700 nm (Annika Maria and Jobi Xavier 2020; New-Lee *et al.*, 2020; Keerthana *et al.*, 2021).

DPPH Assay:

DPPH radical scavenging assay method was done through the addition of radical species that decolourizes the solution. Various concentration of extract (20-120 µg/ml) were taken. 0.1 ml of DPPH solution in 1 ml methanol was added. Incubated at room temperature for 30 min in dark. The absorbance was measured at 517 nm (Ganiyu *et al.*, 2013; Annika Maria and Jobi Xavier, 2020; New-Lee *et al.*, 2020; Keerthana *et al.*, 2021).

Equation to convert the absorbance value into percentage Anti-oxidant activity:

$$\% \text{ Inhibition} = [(B-T) / B] * 100$$

Where B = absorption of blank sample;

T=absorption of test sample

Anti-bacterial Activity:

Microorganism for this study was cultured in Laboratory. They were sub-cultured for studying the separate extract analysis and combinational study. The extract was diluted with MeOH. The plates were prepared with agar medium. Then, five wells were bored in each plate. Samples were loaded in each of the well after marking and kept overnight. Zones were checked for the length. The agar well were dug and analysed (Sathya *et al.*, 2008; Kabesh *et al.*, 2015; Keerthana *et al.*, 2021). Various concentration of the extract (20-120 µg/ml) was taken in clean test tubes. 1ml of blood sample was loaded in 10 ml saline (0.9 g Sodium Chloride (NaCl) in 100 ml). This was centrifuged for 5 min. The supernatant was discarded. The pellet was put in 10 ml saline. Again, centrifugation was carried out for 5 min. The supernatant was discarded. Now, 200 µl saline was added in each test tube. This was then incubated for 1 h at 50°C. The absorbance was read at 560 nm (Sathya *et al.*, 2008; Kabesh *et al.*, 2015; Keerthana *et al.*, 2021).

In vitro Analysis of Combined Extract:

In vitro analysis of the combinational extract has been used with a 3T3-L1 cell line. *In vitro* cell line study was carried for the purpose of cell death analysis. Cell toxicity or cytotoxicity level was analysed from this study with the new combine plant extract as a new marker in the cells that were cultured (Sathya *et al.*, 2008; Keerthana *et al.*, 2021).

Results and Discussion

Phytochemical analysis of *C. roseus* root extract and *P. nigrum* fruit extract was studied. Phenols, tannins, terpenoids, glycosides, flavonoids, and steroids were present in both the extracts (Table 1). The Phenol and Flavonoids need to be present at higher concentrations in order to support for the anti-diabetic activity. So, the concentration of phenols and flavonoids has been checked for the *C. roseus* root extract, *P. nigrum* fruit extract and the combined extract. In the crude extracts, phenol

Table 1: Phytochemical analysis of *C. roseus* root extract and *P. nigrum* fruit extract

Phytochemical	<i>C. roseus</i> root extract	<i>P. nigrum</i> fruit extract
Alkaloids	+	+
Phenols	+	+
Tannins	+	+
Terpenoids	+	+
Glycosides	+	+
Flavonoids	+	+
Saponin	-	-
Steroids	+	+

+ present; - absent

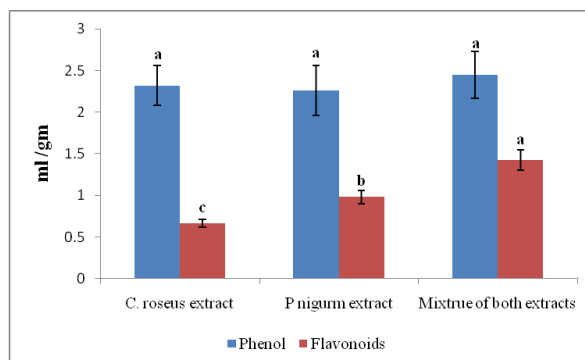


Fig. 1: Concentrations of Phenol and Flavonoids in the *C. roseus* root extract, *P. niger* fruit extract and mixture of both extracts., Values are represented as mean $\pm$ SD (n=6). Different superscripts on column showed significant different ($P \leq 0.05$).

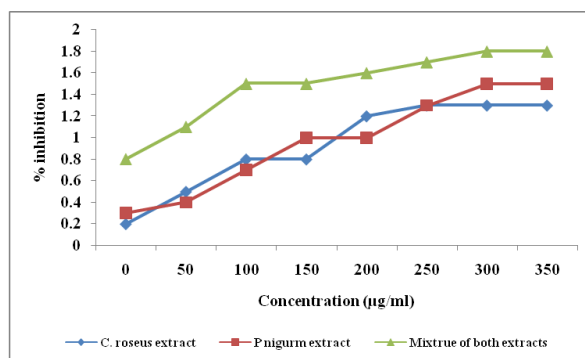


Fig. 2: α - amylase inhibition assay on *C. roseus* root extract, *P. niger* fruit extract and mixture of both extracts.

was present in the range of concentration of 2.26 – 2.45 ml/g, however, flavonoid was lowest concentration in the extracts. The concentration of flavonoid showed significantly higher ($P < 0.05$) in mixture of both extracts (Fig. 1).

The phytochemical analysis was done to find out the presence of various predominant phytochemicals. Saponins were alone found to be absent in all the three extracts namely, *C. roseus* root, *P. nigrum* and mixture of both extracts.

α -amylase inhibition assay was performed to check the anti-diabetic activity. It was found positive for both the crude and the combined extract. Also, the anti-diabetic activity of the *C. roseus* root was found to be greatly enhanced with Piperine as a bio-enhancer in the combined extract (Fig. 2). The Optical Density measurements were found to be continuously increasing for both the extracts. Finally, it was found that the anti-diabetic activity exhibited by *C. roseus* root extract

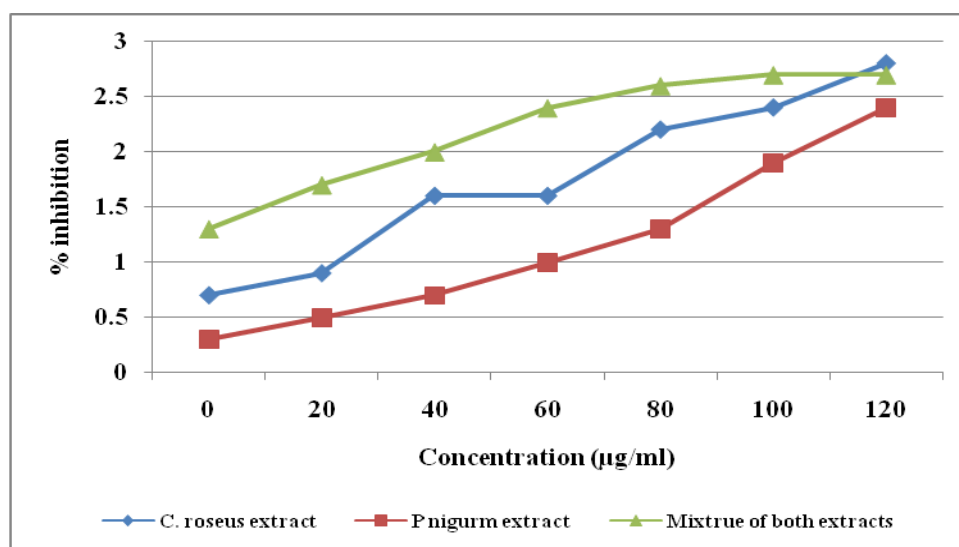


Fig. 3: DPPH assay on *C. roseus* root extract, *P. nigrum* fruit extract and mixture of both extracts.

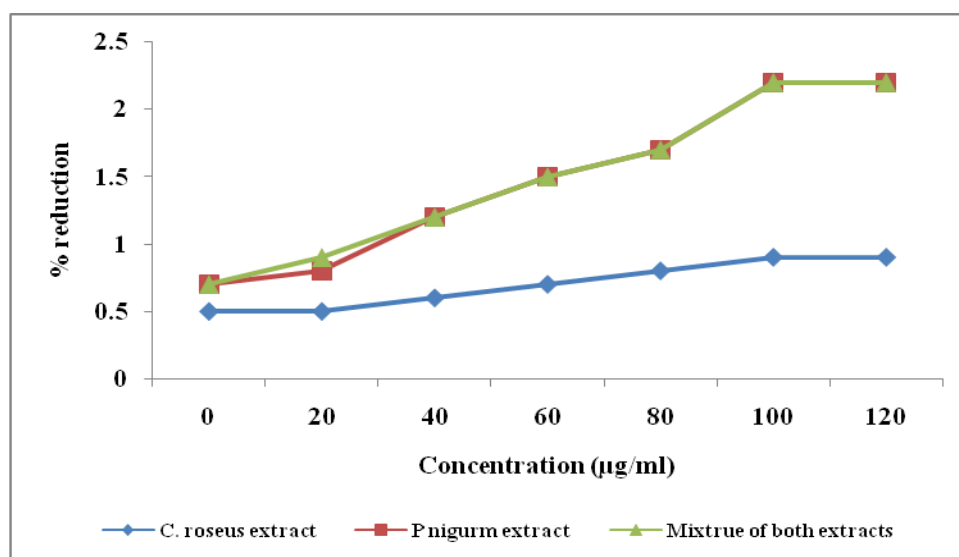


Fig. 4: Effect of *C. roseus* root extract, *P. nigrum* fruit extract and mixture of both extracts on Fe^{3+} reducing power assay.

with Piperine was increased. The decolourized samples with concentrations were studied for absorbance under the DPPH assay. The individual extracts of *C. roseus* roots and *Piper nigrum* fruit extract exhibited good anti-oxidant activity (Fig. 3). But, the combined extract has shown a much better anti-oxidant activity. Fe^{3+} assay was conducted to analyse the anti-oxidant which can confirm the presence of flavinoid which is a natural agent that can help in lowering the risk of heart disease, neurodegenerative disease. The

Optical Density measurements were made to look for the anti-oxidant activity of the *C. roseus* root extracts, *P. nigrum* fruit methanolic extract and the combined crude extract. Figure 4 explains the better anti-oxidant activity of the combined crude extract of *C. roseus* and Piperine. The results of α -amylase, DPPH, Fe^{3+} and anti-inflammatory showed a good increase of inhibition and absorbance factors for the *C. roseus* and *Piper nigrum* fruit combination. The activity is induced with the presence of piperine element from the



Fig. 5: Anti microbial activity of *C. roseus* root extract, *P. nigrum* fruit extract and mixture of both extracts.

Piper nigrum fruit.

Soxhlet extraction method obtained from the extract of *C. roseus* and its phytochemical analysis confirms the presence of Tannins, Flavonoids, Alkaloids, Saponins, Terpenoids, Glycosides by using Methanol. *C.roseus* root extracts were taken for phytochemical analysis. Terpenoids, Flavonoids, Glycosides, Tannins were found to be present. Phytochemical analysis for root extract of *C. roseus* was shown to be absent for Saponins in Methanol (Janet Alejandra *et al.*, 2018).

The Saponins, naturally occurring glycosides and triterpene glycosides in plants tend to cause toxicity in human beings when crossing the tolerable intake, which may induce cytosolic Ca^{2+} activity and trigger erythrocyte membrane scrambling eryptosis and hemolysis can occur at a concentration of 15 $\mu\text{g}/\text{ml}$ which is the associated effects of Saponin increase in blood (Deng *et al.*, 2011; Auyeung *et al.*, 2012; Rosi *et al.*, 2014; Nuanjan *et al.*, 2016). The natural products of terpenoids, alkaloids, flavonoids, phenolics are that which exhibits antidiabetic activity of plant extracts (Mankil *et al.*, 2010; Janet Alejandra *et al.*, 2018).

The recent study on *C. roseus* has shown a remarkable effect on adipogenesis in which it has been reported that $1\alpha, 25$ dihydroxyvitamin D_3

containing fractions of *C. roseus* leaf inhibited lipolysis in 3T3L1 cells. Preadipocytes inhibited lipid accumulation in 3T3L1 cells was reduced significantly using *C. roseus* (Anuj Kumar *et al.*, 2019). Exposing 12.5 $\mu\text{g}/\text{ml}$ *C. roseus* leaf extract for 24 h shows reduction in Fasn gene expression levels to 53% (Gulben *et al.*, 2020). The same gene expression profile of 3T3L1 cells taken at 48 h and 72 h were statistically significant. It is established that *C. roseus* extract fed mice reduces the amount of free fatty acids by decreasing the Fasn (Rasineni *et al.*, 2010).

This study also observed the antibacterial activity for *C. roseus* plant crude extract and Piperine bio-enhancer extract. The combined extract was also tested for antibacterial activity which showed satisfactory results. Anti-bacterial activity was analysed by Agar-well diffusion method. The assay was conducted for the *C. roseus* root extract, *P. nigrum* fruit extract and the mixture of both the extracts. The anti-bacterial activity was proven to be greater when Piperine is employed (Fig. 5).

C. roseus leaf was dissolved in ethanol to reduce the triglycerides and cholesterol level in diabetic mice and pig by feeding them with the extract regularly (Naznin *et al.*, 2009). 120 alkaloids were produced from *C. roseus*; out of 120, 70 alkaloids were pharmacologically active.

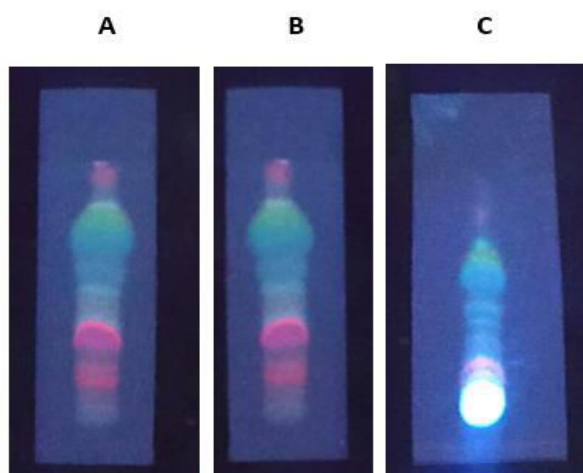


Fig. 6: Thin Layer Chromatography show the presence of alkaloid in (A) *C. roseus* root extract (B) *P. nigrum* fruit extract and (C) mixture of both extracts.

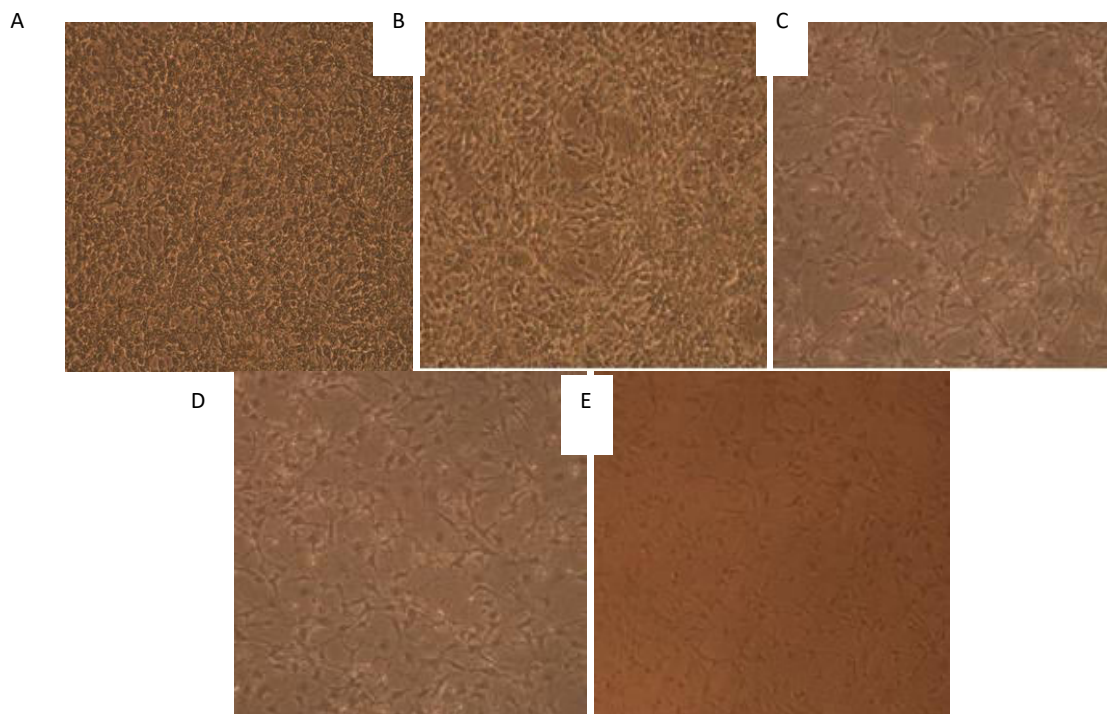


Fig. 7: Optical image of 3T3L1 adipose tissue cell line treated on mixture of both extracts A. Control; B. 25 µg/ml; C. 50 µg/ml; D. 100 µg/ml; E. 250 µg/ml.

These 70 alkaloids can influence different biological effects (Jian Hua *et al.*, 2019). In the present study, Thin Layer Chromatography of three extracts showed presence of alkaloids, however, greater amounts were present in mixture of both the extracts (Fig. 6).

The anti-inflammatory assay performed for the three extracts, *C. roseus* root extract, *P. nigrum* fruit extract and the mixture of the both

extract. This was verified based on the colors specific to particular alkaloids found from previous literature. Applying the combinational extract on 3T3L1 cells for the study of apoptosis, showed an acceptable result. On the other hand, it reduced the lipid accumulation by inducing lipolysis in mature adipocytes (Anuj Kumar *et al.*, 2019). The apoptosis study was carried out on 3T3L1 adipose tissue cell line (Table 2, Fig. 7). The

Table 2: Percentage of cell death of 3T3L1 adipose tissue cell line on mixture of the both extracts

Concentration (µg/ml)	Cell death (%)
Control	0
25	48.92
50	52.65
100	56.21
250	62.57

percentage of mortality was linearly increased corresponding to the concentration of mixture extracts. The LD₅₀ value was 48 µg/ml recorded against the 3T3L1 adipose tissue cell line.

Conclusion

From the results it is concluded that the *C. roseus* extract exhibited a better anti-diabetic activity with Piperine as bioenhancer. As the plants used are also easily available in the Indian subcontinent, it could be used as an effective economic anti-diabetic drug. These plant extracts or decoction could be used as a dietary supplement for diabetic patients for the control and management of diabetes mellitus. Thus, it is proven that the *C. roseus* root extract with Piperine obtained from methanolic extract of *Piper nigrum* as a bio-enhancer could be used as an efficient drug for Diabetes mellitus. The *in vitro* cell line study was carried out in 3T3-L1 cell line, this cell line is a cell line from animal i.e. mouse which is commonly used for biological research on adipose tissues. It has fibro-blast like morphology. This cell line can be used for cellular mechanisms associated with diabetes, obesity and other related disorders. Thin Layer Chromatography was performed to confirm the presence of the required alkaloids namely, Vinblastine. This study's results prove the presence of Piperine as a bioenhancer which proved to improve the anti-diabetic activity of *C. roseus*. This study can be help in future for targeted drug delivery system for enhanced performance.

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