

Comparative *in vitro* Evaluation of the Antioxidant Potential of Apomorphine and Pyrazolones for Hepatocellular Carcinoma

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ABSTRACT

Background: Hepatocellular Carcinoma (HCC) is a common and highly aggressive form of cancer worldwide. Oxidative stress is closely associated with the development and progression of HCC. An excessive increase in Reactive Oxygen Species (ROS) may damage important cellular components, including DNA, lipids, and proteins, through processes such as DNA damage, lipid peroxidation, protein oxidation and dysregulation of cellular signalling pathways. Therefore, compounds possessing antioxidant properties may help mitigate oxidative damage and provide therapeutic benefits in HCC. **Objectives:** This study was undertaken to assess the antioxidant activity of Apomorphine and Pyrazolones using multiple *in vitro* antioxidant assays in HepG2 cells. **Materials and Methods:** Antioxidant activity was determined through a series of *in vitro* assays, including DPPH radical scavenging, Ferric Reducing Antioxidant Power, Thiobarbituric Acid Reactive Substances, Nitric Oxide scavenging and Hydrogen Peroxide (H₂O₂) scavenging assays. HepG2 cells were cultured under standard laboratory conditions and treated with the test compounds. Antioxidant activity was expressed as IC₅₀ values where applicable, Lipid peroxidation was assessed by determining the levels of Malondialdehyde (MDA). The obtained data were statistically investigated using GraphPad Prism software and presented as mean±standard deviation. **Results:** Apomorphine and Pyrazolones exhibited measurable antioxidant activity in multiple assays. In the DPPH assay, Apomorphine demonstrated greater radical scavenging activity (IC₅₀=66.90±0.028 µg/mL) than Pyrazolones (IC₅₀=97.26±0.031 µg/mL). In the FRAP assay, both compounds showed ferric reducing activity with IC₅₀ values of 4.822±0.247 µg/mL and 4.127±0.832 µg/mL, respectively. TBARS analysis revealed lower MDA levels in the Apomorphine-treated group (4.615 nmoles/mL) compared with the Pyrazolones-treated group (6.795 nmoles/mL), indicating reduced lipid peroxidation. Both compounds also exhibited nitric oxide scavenging activity. However, the IC₅₀ values obtained for both compounds in the hydrogen peroxide scavenging assay exceeded the highest concentration tested. **Conclusion:** The findings indicate that Apomorphine and Pyrazolones possess significant antioxidant properties with Apomorphine demonstrating comparatively stronger antioxidant activity in several evaluated parameters. These results suggest that both compounds may contribute to the regulation of oxidative stress and warrant further investigation to elucidate their potential biological significance in hepatocellular carcinoma.

Keywords: Antioxidant activity, DPPH, FRAP, Hydrogen Peroxide, Nitric oxide, Oxidative stress, TBARS.

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Received: 15-06-2026;

Revised: 03-08-2026;

Accepted: 24-09-2026.

INTRODUCTION

Hepatocellular Carcinoma (HCC) represents a major global health concern and contributes substantially to the burden of liver cancer (Kudo *et al.*, 2023; Llovet *et al.*, 2021). Its pathogenesis involves complex interactions among cell-cycle dysregulation,

immune escape mechanisms and oxidative stress-induced damage. The emergence of CDK4/6 inhibitors and immune checkpoint modulators has revolutionized targeted therapy for HCC by providing dual-action tumour suppression through cell-cycle arrest and immune system reactivation. However growing evidence suggests that Oxidative stress contributes significantly to the progression of tumours, thereby necessitating antioxidant strategies to mitigate Reactive Oxygen Species (ROS)-driven carcinogenesis. Ayurvedic and Unani medical systems have historically recognized the therapeutic benefits of herbal compounds in preventing and managing cancer. The Charaka Samhita (1000 B.C.) is one of the earliest texts documenting



DOI: 10.5530/ijpi.20270134

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over 2,000 medicinal plants, many of which are known to exhibit antioxidant, anti-inflammatory and antineoplastic properties (Kudo *et al.*, 2023; Llovet *et al.*, 2021; Charaka, 1000 B.C.). Among these, *Oxalis corniculata* Linn. (Family: Oxalidaceae) has gained attention for its broad-spectrum biological activities, including hepatoprotective, immunomodulatory and free radical-scavenging properties (Unani Medical Council, 2020; Singh and Patel, 2019; Halliwell, 2022). Plant-derived antioxidants play a pivotal role in reducing oxidative damage, which is a known contributor to cancer, atherosclerosis, cardiovascular diseases and inflammation (Rojo de la Vega *et al.*, 2018; George *et al.*, 2022; Finn *et al.*, 2015). The accumulation of ROS can lead to DNA mutations, lipid peroxidation and protein degradation, thereby exacerbating tumour development and progression (Parker and Finn, 2023; Cai *et al.*, 2024). Therefore, integrating antioxidant-rich compounds with modern therapeutic strategies may enhance the overall efficacy of cancer treatment. The regulation of free radicals in hepatic cells involves a delicate balance between enzymatic and non-enzymatic antioxidant systems, including Superoxide Dismutase (SOD), Catalase and glutathione (Qiu *et al.*, 2024; Zamio, 2024; Beaufrère *et al.*, 2024). The nuclear transcription factor Nrf2 plays a crucial role in the regulation of antioxidant genes and the maintenance of cellular redox homeostasis. To assess the antioxidant potential of the investigated compounds, the present study employed five key antioxidant assays, namely the DPPH assay for determining free radical scavenging efficiency, the FRAP assay for measuring ferric reducing antioxidant power, the TBARS assay for assessing lipid peroxidation levels, the nitric oxide assay for evaluating nitric oxide radical scavenging capacity and the hydrogen peroxide assay for assessing ROS scavenging activity and oxidative damage control. CDK4/6 inhibitors such as Palbociclib suppress uncontrolled cellular proliferation by inducing G1-phase cell-cycle arrest, thereby restricting tumour progression (He *et al.*, 2021; Zhang *et al.*, 2023; Wu *et al.*, 2023; He *et al.*, 2021). Simultaneously, immune checkpoint restores T-cell-mediated immune responses and counteract immune evasion strategies employed by HCC cells modulators focusing on the PD-1/PD-L1 and CTLA-4 pathways (Toyokuni and Akatsuka, 2020; Brand-Williams *et al.*, 1995; Benzie and Strain, 1996; Ohkawa *et al.*, 1979). Given the intricate relationship among redox homeostasis, immune activation, and cell-cycle regulation, evaluating antioxidant parameters alongside targeted therapeutic approaches may provide valuable insights into improving treatment outcomes in HCC. The primary objective of the present study was to evaluate the antioxidant profile of Apomorphine and Pyrazolones in HepG2 cells and to analyse the regulation of oxidative stress using DPPH, FRAP, TBARS, nitric oxide and hydrogen peroxide scavenging assays. The ongoing study may contribute to a better understanding of the antioxidant properties of these compounds and their potential relevance to oxidative stress modulation in hepatocellular carcinoma.

MATERIALS AND METHODS

Materials

Cell Culture

The human hepatocellular carcinoma cell line was procured from the National Center for Cell Science (NCCS), Pune, India. The cells were set in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin solution. The cultures were incubated at 37°C in a humidified atmosphere comprising 5% CO₂. HepG2 cells are extensively used as an *in vitro* model for Hepatocellular carcinoma research due to their stable hepatic characteristics and reproducible growth pattern (Llovet *et al.*, 2021).

Drug Treatment

Apomorphine and Pyrazolones were procured from a commercial source and evaluated for their antioxidant potential in the present study. Unless otherwise specified, Pyrazolones were dissolved in sterile distilled water, whereas Apomorphine was dissolved in Dimethyl Sulfoxide (DMSO). Equal volumes of solvent were maintained in the control groups and the final concentration of DMSO in the culture medium did not surpass 0.1% (v/v). All experimental solutions were freshly prepared and sterilized using 0.22 µm syringe filters before use.

Treatment Protocol

Depending on the experimental assay, cells were seeded in either 6-well or 96-well plates and allowed to attach for 24 hr before being treated with various concentrations of Apomorphine or Pyrazolones dissolved in DMSO or distilled water. Control cells received the same volume of the vehicle solution and the highest concentration of DMSO remained below 0.1% (v/v).

Methods

DPPH Assay

The test substances antioxidant activity was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging test. For the test, 100 µL of a methanol-prepared 0.1 mM DPPH solution was added to a 96-well microplate along with 5 µL of the test compounds at the respective concentrations. Methanol used for the assay was procured from SD Fine Chemicals. Ascorbic acid served as the reference antioxidant. All concentrations were evaluated in quadruplicate ($n=4$), and the results were specified as mean ± Standard Deviation (SD). Blank wells were prepared by adding 200 µL methanol and 10 µL of test compound or standard without DPPH. Wells containing only DPPH solution served as the control, whereas wells without DPPH were considered blank. A combination of reactions containing 20 µL deionized water was used as the negative control (Abdulrasheed-Adeleke and Musa, 2020). The microplate was kept at room temperature in the dark for 30 min before recording the absorbance at 517 nm with a microplate reader. DPPH radical inhibition was

expressed as a percentage relative to the control. The IC₅₀ values were obtained using GraphPad Prism 6 software by plotting the test concentration along the X-axis against the corresponding percentage inhibition on the Y-axis (Imam *et al.*, 2011).

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was carried out to determine the declining ability of the test compounds. Briefly, 40 µL of 0.2 M sodium phosphate buffer, 50 µL of 1% potassium ferricyanide [K₃Fe(CN)₆] and 10 µL of various concentrations of the test compounds or standard ascorbic acid were added into a 96-well plate. After mixing, the reaction mixture was incubated at 50°C for 20 min. Untreated wells containing the reaction mixture without test compounds were used as controls. Subsequently, 50 µL of 10% trichloroacetic acid was added to each well. To develop the colour response, 50 µL deionized water and 50 µL of 0.1% ferric chloride solution were mixed according to the standard FRAP protocol (Rao *et al.*, 2013). Absorbance was calculated at 700nm through a microplate reader. Anti-oxidant activity was expressed as IC₅₀ values obtained using GraphPad Prism 6 software. A dose-response curve was plotted between concentration and absorbance (Xavier and Reddy, 2013). Each measurement was carried out in quadruplicate (*n*=4) and the results are indicated as mean±SD.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

Lipid peroxidation was evaluated using the TBARS assay. Approximately 50 mg of HepG2 cell pellet obtained after treatment was homogenized in 1 mL Phosphate-Buffered Saline (PBS) to prepare the test solution. From the homogenate, 100 µL was mixed with 500 µL of Solution A and vortexed thoroughly. The mixture was centrifuged at 2500 rpm for 5 min and the supernatant was obtained. Subsequently, 1800 µL of Thiobarbituric Acid (TBA) reagent was added and mixed thoroughly. The reaction combination was heated in a water bath for 15 min to facilitate colour development. Absorbance was recorded at 532 nm against a reagent blank using a spectrophotometer or microplate reader. The concentration of Malondialdehyde (MDA), an indicator of lipid peroxidation, was assessed using the molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ (Olszewska-Slonina *et al.*, 2011). All measurements were performed in quadruplicate (*n*=4) and the results were expressed as mean±SD.

Nitric Oxide (NO) Scavenging Assay

Nitric oxide scavenging activity was assessed by following the sodium nitroprusside method. For the reaction, 50 µL of 10 mM sodium nitroprusside was combined with 40 µL of distilled water and 10 µL of the test sample, standard, or blank solution. Untreated wells served as controls. The combinations were kept at ambient temperature and exposed to light for 15 min to facilitate nitric oxide generation. Thereafter, 100 µL of Griess reagent was included to each well and incubated for an additional 5-10 min

for chromophore development. Absorbance was measured at 540 and 660 nm using a microplate reader. The percentage inhibition of nitric oxide radicals was computed relative to the management. IC₅₀ values were calculated via GraphPad Prism 6 software (Rao *et al.*, 2013). Each experiment was carried out in quadruplicate (*n*=4), and the data are presented as mean±SD.

Hydrogen Peroxide (H₂O₂) Scavenging Assay

The hydrogen peroxide scavenging efficacy of the test compounds was evaluated using a colorimetric method. In a 96- well plate, 10 µL of test sample or standard was added into designated wells. Wells in SET 1 and SET 3 received 90 µL of 6% hydrogen peroxide solution prepared in distilled water, whereas SET 2 and SET 4 received 90 µL buffer solution. The plate was incubated at 37°C for 5 min according to previously reported methods (Rahman *et al.*, 2015). Following incubation, 100 µL of ammonium molybdate reagent was included to each well. The plates were then incubated at 37°C for 2 min to allow development of the chromogenic response. Absorbance was recorded at 405-415 nm using a microplate reader. Hydrogen peroxide scavenging was expressed as percentage inhibition relevant to the control. The IC₅₀ values were estimated using GraphPad Prism 6 software by plotting the concentration against the corresponding percentage inhibition (Hazra *et al.*, 2008). Measurements were carried out in quadruplicate (*n*=4), and the data are presented as mean±SD.

Statistical Analysis

Each experiment was directed using four replicates (*n*=4), and the findings are reported as mean±Standard Deviation (SD). GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. An unpaired Student's t-test was used to compare the two groups. One-way variance analysis (ANOVA) and Tukey's *post hoc* test were employed when comparing more than two groups. When the *p*-value was less than 0.05, differences were deemed statistically significant.

RESULTS

DPPH Radical Scavenging Activity

The antioxidant activity of Apomorphine and Pyrazolones was assessed utilizing the DPPH radical scavenging test and contrasted with ascorbic acid, a common antioxidant. The IC₅₀ values of ascorbic acid, Pyrazolones, and Apomorphine were found to be 19.25±0.023 µg/mL, 97.26±0.031 µg/mL and 66.90±0.028 µg/mL, respectively. Among the tested compounds, Apomorphine exhibited a lower IC₅₀ value than Pyrazolones, indicating comparatively greater DPPH radical's detoxification activity (Figure 1).

Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reduction antioxidant activity of Apomorphine and Pyrazolones was assessed using the FRAP assay. The IC₅₀

values of ascorbic acid, Pyrazolones and Apomorphine were $2.258 \pm 0.023 \mu\text{g/mL}$, $4.127 \pm 0.832 \mu\text{g/mL}$ and $4.822 \pm 0.247 \mu\text{g/mL}$. Both compounds demonstrated ferric reducing activity under the experimental conditions employed. The results are shown in Figure 2.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

Lipid-peroxidation was evaluated via measuring Malondialdehyde (MDA) levels by using TBARS assay. The MDA concentrations observed in the Pyrazolones- and Apomorphine-treated groups were 6.795 nmoles/mL and 4.615 nmoles/mL, respectively. A lower MDA level was observed in the Apomorphine-treated group compared with the Pyrazolones-treated group. The results are presented in Figure 3.

Nitric Oxide (NO) Scavenging Activity

The nitric oxide scavenging activity of Apomorphine and Pyrazolones was determined and assessed with gallic acid as the

reference standard. The IC_{50} values of gallic acid, Pyrazolones and Apomorphine seemed $18.96 \pm 0.026 \mu\text{g/mL}$, $417.9 \pm 0.047 \mu\text{g/mL}$ and $1844 \pm 0.110 \text{ ng/mL}$, respectively. Both compounds demonstrated nitric oxide scavenging activity within the tested concentration range. The results are presented in Figure 4.

Hydrogen Peroxide (H_2O_2) Scavenging Activity

The H_2O_2 activity of Apomorphine and Pyrazolones was evaluated utilizing the hydrogen peroxide radical scavenging test and contrasted with gallic acid as the reference typical. Gallic acid exhibited an IC_{50} value of $84.16 \pm 0.020 \mu\text{g/mL}$. However, the IC_{50} values of both Apomorphine and Pyrazolones could not be determined within the tested concentration range and were therefore recorded as above the maximum tested dose. These results suggest limited hydrogen peroxide scavenging activity of both compounds under the experimental conditions employed. The results are presented in Figure 5.

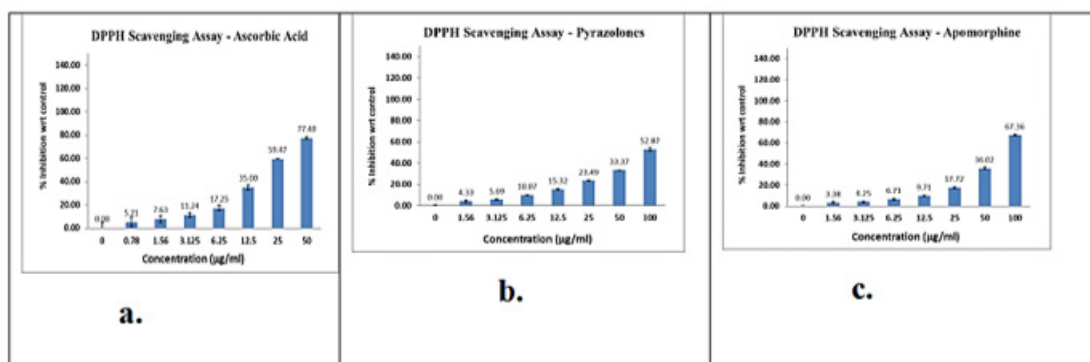


Figure 1: Dose-dependent DPPH radical scavenging activity of Ascorbic Acid, Pyrazolones and Apomorphine. The graph depicts % inhibition versus concentration ($\mu\text{g/mL}$). IC_{50} values were calculated using non-linear regression analysis (GraphPad Prism 6). Ascorbic acid showed the most potent activity with the lowest IC_{50} value, followed by Apomorphine and Pyrazolones.

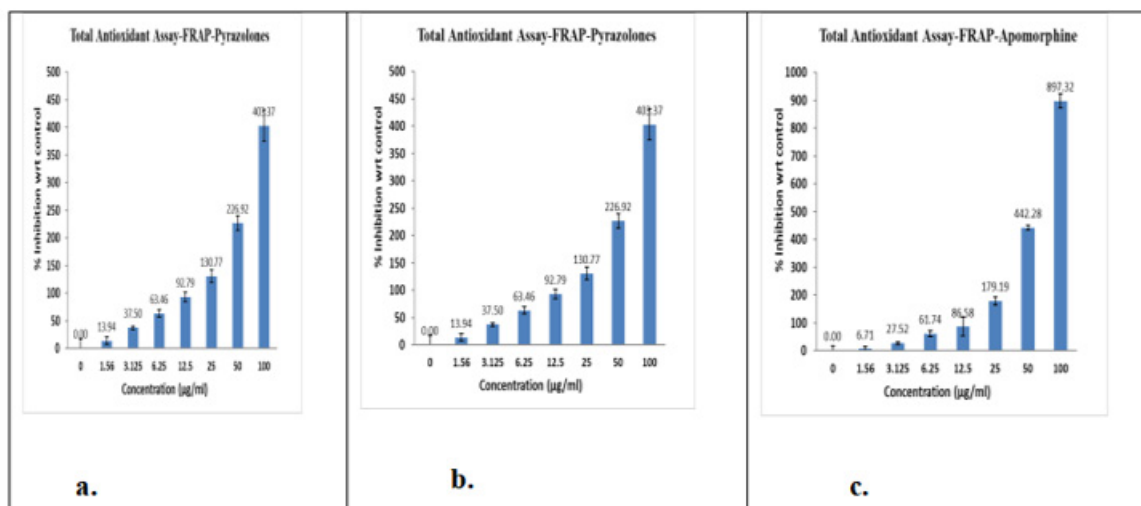


Figure 2: a. Ferric Reducing Antioxidant Power of Ascorbic Acid at various concentrations (0-50 $\mu\text{g/mL}$). A strong dose-dependent increase in antioxidant activity was observed. $\text{IC}_{50}=2.258 \pm 0.023 \mu\text{g/mL}$. b. FRAP activity of Pyrazolones at different concentrations (0-100 $\mu\text{g/mL}$). The reducing power increased with concentration, reaching 50% inhibition at $4.127 \pm 0.832 \mu\text{g/mL}$. c. FRAP activity of Apomorphine at different concentrations (0-100 $\mu\text{g/mL}$). The compound showed moderate reducing ability with an IC_{50} value of $4.822 \pm 0.247 \mu\text{g/mL}$.

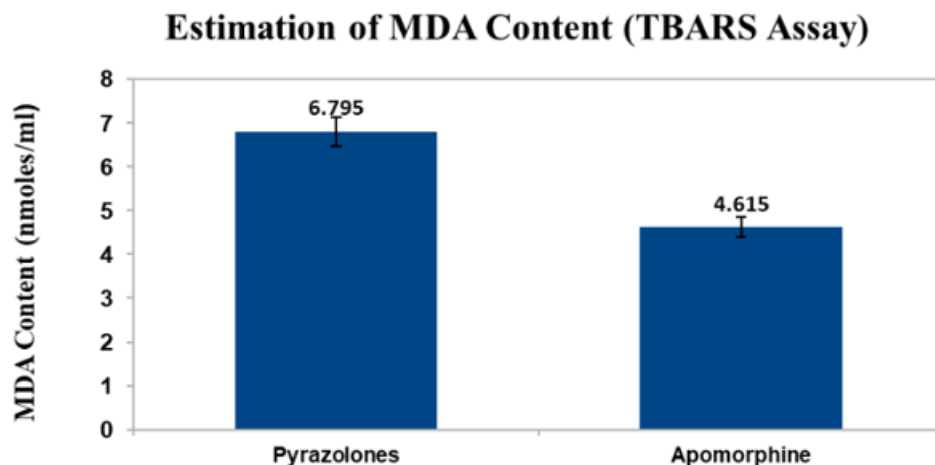


Figure 3: Comparison of MDA content (nmoles/mL) between Pyrazolones and Apomorphine samples as measured by TBARS assay. Pyrazolones showed a higher lipid peroxidation level than Apomorphine, indicating increased oxidative stress.

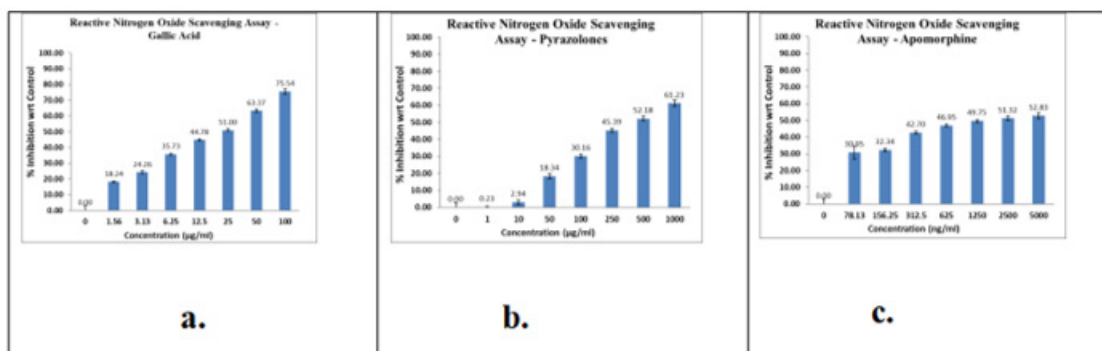


Figure 4: a. Nitric oxide scavenging activity (% inhibition) of Gallic Acid at different concentrations (0-100 µg/mL). The results are expressed as mean percentage inhibition relative to control. b. Nitric oxide scavenging activity (% inhibition) of Pyrazolones at various concentrations (0-1000 µg/mL). A dose-dependent increase in radical scavenging was observed. c. Nitric oxide scavenging activity (% inhibition) of Apomorphine at different concentrations (0-5000 ng/mL). The activity was moderate and dose-responsive but comparatively lower than that of Pyrazolones and Gallic Acid.

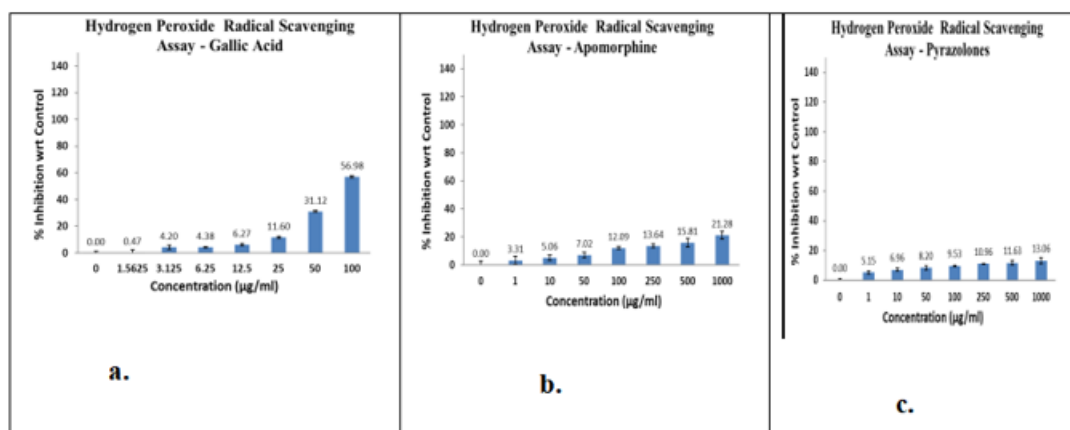


Figure 5: a. Hydrogen peroxide scavenging activity (% inhibition) of Gallic Acid at various concentrations (0-100 µg/mL). The standard exhibited a strong dose-dependent increase in inhibition with an IC_{50} of 84.16 µg/mL. b. Hydrogen peroxide scavenging activity (% inhibition) of Apomorphine across the tested concentration limit (0-1000 µg/mL). The activity was significantly lower than the standard and no 50% inhibition was achieved. c. Hydrogen peroxide scavenging activity (% inhibition) of Pyrazolones across the tested concentration limit (0-1000 µg/mL). Similar to Apomorphine the activity remained below the 50% inhibition threshold.

DISCUSSION

Oxidative stress is recognized as an important contributor to the development and progression of Hepatocellular Carcinoma (HCC). Excessive production of Reactive Oxygen Species (ROS) can induce DNA damage, lipid peroxidation, protein oxidation and alterations in cellular signalling pathways, thereby promoting tumour progression. Consequently, the identification of compounds with antioxidant properties may provide valuable support in strategies aimed at reducing oxidative stress-associated cellular damage.

In the present study, the antioxidant potential of Apomorphine and Pyrazolones was evaluated using multiple *in vitro* antioxidant assays, including DPPH, FRAP, TBARS, nitric oxide and hydrogen peroxide scavenging assays. The use of multiple assays was important because antioxidant activity is a complex phenomenon and no single method can comprehensively evaluate all antioxidant mechanisms. The DPPH assay demonstrated that both compounds possessed free radical scavenging activity with Apomorphine exhibiting greater activity than Pyrazolones. The DPPH method primarily measures the capacity of a compound to donate electrons or hydrogen atoms to neutralize free radicals. Similar observations have been reported in previous studies, where compounds with effective electron-donating properties showed enhanced DPPH radical scavenging capacity (Brand-Williams *et al.*, 1995; Halliwell, 2022).

The FRAP assay further confirmed the antioxidant potential of both compounds by demonstrating their ferric ion reducing capacity. Since the FRAP assay is based on electron transfer reactions rather than direct radical scavenging, differences between DPPH and FRAP results are expected. Previous studies have shown that antioxidant compounds may perform differently across assays because each method evaluates distinct mechanisms of antioxidant action (Benzie and Strain, 1996). The results obtained in the present study support this observation and highlight the importance of employing multiple antioxidant assays for comprehensive evaluation.

Lipid peroxidation is a major consequence of oxidative stress and contributes to membrane damage and cellular dysfunction. The TBARS assay revealed lower Malondialdehyde (MDA) levels in the Apomorphine-treated group compared with the Pyrazolones-treated group, indicating reduced lipid peroxidation. Since MDA is a widely accepted biomarker of oxidative damage, the observed findings suggest that Apomorphine may provide greater protection against oxidative membrane injury under the experimental conditions employed (Ohkawa *et al.*, 1979; Rojo de la Vega *et al.*, 2018).

Nitric oxide scavenging analysis demonstrated that both compounds were capable of interacting with reactive nitrogen species. Nitric oxide plays an important role in inflammation and oxidative stress and excessive production has been associated with

cancer progression and tissue injury. The observed scavenging activity therefore indicates an additional antioxidant property of the tested compounds. Similar findings have been reported for several natural and synthetic antioxidants capable of reducing nitric oxide-mediated oxidative damage (Rao *et al.*, 2013).

In contrast, both Apomorphine and Pyrazolones exhibited limited activity in the hydrogen peroxide scavenging assay, as their IC₅₀ values could not be determined within the tested concentration range. This finding highlights the assay-dependent nature of antioxidant activity, where a compound may demonstrate effectiveness against certain reactive species while exhibiting limited activity against others. Such differences are commonly observed because individual antioxidants interact differently with specific oxidants and free radical systems (Hazra *et al.*, 2008; Rahman *et al.*, 2015).

Overall, the variations observed among DPPH, FRAP, TBARS, nitric oxide and hydrogen peroxide assays emphasize that antioxidant activity involves multiple mechanisms, consisting of minimizing power, neutralizing radicals and preventing lipid peroxidation. Collectively, the results demonstrate that both Apomorphine and Pyrazolones possess measurable antioxidant properties with Apomorphine demonstrating comparatively stronger antioxidant activity in several of the evaluated parameters. Further studies are necessary to investigate the underlying molecular mechanisms and to determine the potential biological significance of these findings in hepatocellular carcinoma models.

CONCLUSION

In conclusion, Apomorphine and Pyrazolones exhibited measurable antioxidant activity in multiple *in vitro* assays, including DPPH, FRAP, TBARS and nitric oxide scavenging assays. Among the tested compounds, Apomorphine demonstrated comparatively stronger antioxidant activity in several evaluated parameters. These findings suggest that both compounds possess antioxidant properties that may contribute to the modulation of oxidative stress. Future investigations involving mechanistic studies, ROS analysis and *in vivo* validation are required to establish their potential role in hepatocellular carcinoma and related oxidative stress-associated conditions.

ACKNOWLEDGEMENT

We sincerely thank Teerthankar Mahaveer College of Pharmacy, Teerthankar Mahaveer University, Moradabad for their support. Special appreciation to Krishana Kumar Sharma for his invaluable guidance throughout the research.

ABBREVIATIONS

CDK4/6: Cyclin-Dependent Kinase 4 and 6; **HCC:** Hepatocellular Carcinoma; **Rb:** Retinoblastoma Protein; **DMSO:** Dimethyl Sulfoxide; **FBS:** Fetal Bovine Serum;

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; **MMP:** Mitochondrial Membrane Potential; **PBS:** Phosphate-Buffered Saline; **TBARS:** Thiobarbituric Acid Reactive Substances; **MDA:** Malondialdehyde; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **FRAP:** Ferric Reducing Antioxidant Power; **NO:** Nitric Oxide; **H₂O₂:** Hydrogen Peroxide; **ROS:** Reactive Oxygen Species; **IC₅₀:** Half-Maximal Inhibitory Concentration; **Nrf2:** Nuclear Factor Erythroid 2-Related Factor 2; **PD-1/PD-L1:** Programmed Cell Death Protein 1/Programmed Cell Death Ligand 1; **CTLA-4:** Cytotoxic T-Lymphocyte-Associated Protein 4.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL STATEMENT

HepG2 human hepatocellular carcinoma cell lines used in the present investigation were obtained from the National Center for Cell Science (NCCS), Pune, India. The study was carried out exclusively under *in vitro* conditions and did not involve any human subjects or experimental animals; therefore, ethical committee approval was not applicable for this work.

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Cite this article: Barti H, Sharma KK. Comparative *in vitro* Evaluation of the Antioxidant Potential of Apomorphine and Pyrazolones for Hepatocellular Carcinoma. *Int. J. Pharm. Investigation*. 2027;17(1):322-8.