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Title: Hepatoprotective Activity of Gentisic Acid on 5-Fluorouracil Induced Hepatotoxicity in Wistar Rats

Rohini Revansiddappa Pujari[1], Deepti Dinesh Bandawane[2]
[1]Department Of Pharmacology, Modern College Of Pharmacy (for Ladies), Moshi, Pune-412105, Maharashtra, India.
[2]Department Of Pharmacology, Modern College Of Pharmacy, Yamunanagar, Nigdi, Pune-412105, Maharashtra, India.

Corresponding Author
Rohini Revansiddappa Pujari
rohinirpujari@gmail.com
orcid.org/0000-0003-4169-6094
08087620078
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ABSTRACT
Introduction: 5-Fluorouracil (5-FU) is a very potent and efficacious antineoplastic drug which is used in the management of several types of cancer but its clinical use is associated with severe toxicities including hepatotoxicity which limits its therapeutic value as a potent anticancer agent. **Objective:** The present investigation was carried out to evaluate the hepatoprotective activity of a plant phenolic acid i.e. gentisic acid (2,5-dihyroxybenzoic acid) against hepatotoxicity by 5-FU in Wistar rats. **Method:** The rats were segregated into six groups with six rats in each group. Amongst these, Group I and II served as normal control and 5-FU control groups, respectively and received distilled water (1 ml/kg) for 14 days by oral route. Groups III, IV, V and VI served as test groups and received gentisic acid (GA) at the doses 3, 10, 30 and 100 mg/kg orally for 14 days. On the 9th day, all the groups except group I were administered with 5-FU (20 mg/kg) intraperitoneally which was continued further for the next five days up to 14th day. The rats were sacrificed at the end of the experimental period, blood was withdrawn for biochemical estimations and hepatic tissues were dissected out for histopathological studies. **Results:** Exposure to 5-FU at a dose of 20 mg/kg produced a significant increase in serum hepatic biomarkers such as levels of alanine aspartate aminotransferase (AST), aminotransferase (ALT), alkaline phosphatase (ALP), direct bilirubin (DB), total bilirubin (TB) and total protein content (TPC) along with severe histopathological changes in hepatic tissues of rats indicating severe hepatotoxicity. Pre and co-administration of GA with 5-FU at 30 and 100 mg/kg for 14 days resulted in a dose-dependent amelioration of altered biochemical and histopathological parameters. **Conclusion:** The results indicated the potential of GA as a hepatoprotective agent for the prevention of 5-FU induced hepatotoxicity.
Key Words: 5-Fluorouracil, Gentisic acid, 2,5-Dihyroxybenzoic acid, Hepatotoxicity, Plant phenolics

INTRODUCTION

Cancer chemotherapy-induced hepatotoxicity and impairment of liver function is a significant and frequently appearing adverse clinical complication that often requires dose reduction or withdrawal of chemotherapeutic agents, thereby limiting their therapeutic potential as efficacious anti-neoplastic agents. This negative aspect narrows the extent of clinical application of cancer chemotherapeutic agents regardless of their contribution to the improvement of survival rate.[1,2] 5-Fluorouracil (5-FU) is a fluorinated pyrimidine analogue, which is widely used in the management of various types of cancers such as stomach, breast, head and neck, colorectal, and genitourinary cancers.[3] It is used either alone or in combination. 5-FU acts by incorporating into DNA as well as RNA. Deoxyuridine triphosphate (dUTP) and fluorodeoxyuridine triphosphate (FdUTP) get incorporated into DNA by replacing reduced thymidine triphosphate (TTP) in the cells treated with 5-FU.[4] Incorporation of fluorodeoxyuridylate and deoxyuridylate in DNA causes the initiation of the process of excision-repair, resulting into breakage of DNA strand in the absence of TTP produced due to blockade of enzyme thymidylate synthase. The insertion of 5-FU into RNA causes severe effects on functioning as well as the processing of RNA resulting in severe toxicities.[5] However 5-FU chemotherapy has been reported to often exhibit severe systemic toxicities including hepatotoxicity in clinical practice.[6] 5-FU majorly gets eliminated by hepatic metabolism. Dihydropyrimidine dehydrogenase (DPD) enzyme found in the liver acts as the key enzyme involved in the rate-limiting step of 5-FU catabolism. Toxic intermediates produced during the metabolism of 5-FU play an important role in liver injury.[7] Various *In vitro and In vivo* studies have demonstrated that administration of 5-FU leads to the generation of oxidative stress in the liver which consequently results in structural and functional disruption of hepatocytes. Hence an attempt to reduce the hepatotoxicity of 5-FU may prove to be a vital approach in improvement of chemotherapeutic outcomes.[8-10]

Plants have been utilized as medications to cure various types of ailments and with satisfactory outcomes since the primordial era. In the current scenario, a lot of importance is given to the utilization of phytoconstituents with anti-apoptotic, antioxidant and anti-inflammatory properties to reduce the cancer chemotherapy-induced drug toxicities.[11,12] It has been documented in various preclinical studies that consumption of fruits and vegetables in the daily diet decreases the risk of neoplasm because of the various essential nutrients predominantly phenolics present in them.[13] Phenolic acids are extensively spread all over the plant kingdom embodying about 10,000 varieties of phenolic structures. These compounds are majorly investigated and proved worldwide through several preclinical studies for their valuable outcomes in concern of human well-being.[14,15]

Gentisic acid (GA) has been scientifically reported for its variety of pharmacological actions which include analgesic, antiarthritic and anti-inflammatory activities, antimutagenic and anticancer activities, antirheumatic and antispasmodic activities, antioxidant, antiparkinsonian activity, antifungal activity, iron chelating and siderophoric activities, anti-hyperlipidemic activity, *In vivo* and *In vitro* antioxidant effect, protective activity against cyclophosphamide-induced genotoxicity, inhibitory activity against fibroblast growth factor (FGF) etc.[16-28] GA has also been documented for its protective activity against cyclophosphamide-induced hepatotoxicity wherein pre- and co-treatment with GA significantly ameliorated the increase in malondialdehyde levels produced by cyclophosphamide and normalized all oxidative stress biomarkers including glutathione peroxidase, glutathione reductase, glutathione, catalase and

quinone reductase levels with the reduction in DNA fragmentation and formation of micronuclei at the doses of 50 mg/kg and 100 mg/kg. GA also exhibited a significant reduction in hepatic biomarkers such as alanine aminotransferase, lactate dehydrogenase and aspartate aminotransferase which were significantly increased after administration of cyclophosphamide.[29] But GA has not yet been evaluated for its protective activities against the hepatotoxicity induced by any of the anticancer agents including 5-FU.

The present investigation thus was conducted with an aim to evaluate the protective activity of GA against 5-FU-induced hepatotoxicity by virtue of the quantitative analyses of enzymes of hepatic function in the serum and histopathological investigations to evaluate the ultrastructural alterations in the hepatic tissue.

METHODOLOGY

Chemicals and kits

Gentisic acid was procured from Sigma-Aldrich Chemicals, USA. 5-Fluorouracil (Fiveflurd) was purchased from GlaxoSmithKline Pharmaceuticals Ltd. Other solvents and chemicals utilized were of analytical grade and procured from standard commercial suppliers. Standard commercial diagnostic kits namely Biolab Diagnostics Pvt. Ltd. for biochemical estimations were purchased from Kiran Enterprises, Pune, India.

Animals

Adult Wistar rats of either sex (200-250 g) were used in the present investigation. They were purchased from National Institute of Biosciences (NIB), Pune, Maharashtra and caged in groups of 5-6 rats in standard cages made up of polypropylene with a wire mesh lid and maintained at standard environmental conditions at a temperature of 25 ± 2^0C and 45 to 55% of relative humidity under 12 h light: 12 h dark cycle in the institutional animal house. The animals were provided with free access to standard pelleted food (Nutrivet Life Sciences, Pune, India). All the experiments were carried out between 12:00-16:00 hour. The animals were transferred from animal house to the experimental laboratory one hour before the start of the experiment.

Ethical clearance

All the studies were carried out in compliance with the Institutional Animal Ethical Committee (IAEC) guidelines given as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India (Section 15 of the Prevention of Cruelty to Animals Act, 1960; Ministry of environment and forest (AWD), Government of India). The protocol was approved by IAEC of Modern College of Pharmacy, Yamunanagar Nigdi, Pune-411044. (Proposal No.: MCP/IAEC/004/2017; dated 07/11/2017).

Preparation of drug solutions

GA was accurately weighed at different quantities and dissolved in distilled water to obtain the respective stock solutions of 3, 10 30 and 100 mg/ml. Similarly, 20 mg/ml stock solution of 5-fluorouracil was prepared in distilled water. Appropriate stock solutions were selected for administration of the doses.

Experimental design

The rats were segregated into six groups with each group comprising of six rats. Group I and II served as normal control and 5-FU control groups respectively and received distilled water (1 ml/kg) orally for 14 days. Groups III, IV, V and VI served as test groups and received GA at doses of 3, 10, 30 and 100 mg/kg orally for 14 days. On the 9th day, all the groups except group I were administered 5-FU (20 mg/kg) intraperitoneally which was continued further for the next five days up to 14th day.
The rats were sacrificed by cervical dislocation at the end of the experimental period. Blood was then withdrawn by cardiac puncture into serum separation tubes and by centrifuged at 3000 rpm for 20 min to obtain the serum. The separated serum was then stored into eppendorf tubes at -20^{0}C to be used for the assessment of hepatic biomarkers. The livers were then excised and washed using ice-cold saline solution, dried and then frozen until used for analysis.[29]

Estimation of hepatic biomarkers
Serum levels of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), direct bilirubin (DB) and total protein content (TPC) were determined by spectrophotometry with UV-2600 UV-VIS spectrophotometer (Shimadzu Corporation) using standard biochemical estimation kits.

Histopathological studies
The whole intact livers were fixed by placing them for 24 hours in formalin (10%, v/v) and then they were embedded in paraffin. The representative coronal slices (thickness of 5µm) of organs were cut using a rotary microtome (Biocraft) and stained successively, first with hematoxylin for 8 min and then with eosin for 3 min (Luna et al., 1960). The thin sections were then prepared into permanent slides and observed under 45X magnification power through a digital trinocular microscope (Olympus CX-21-TR) with the photographic facility. The photomicrographs were captured with the help of Magnuspro eyepiece camera software.[30]

STATISTICAL ANALYSIS
The results were expressed as mean $\pm$ SEM. Comparison between the groups was made by one-way analysis of variance (ANOVA) followed by Tukey's Kramer Multiple Comparison test using Instat Graph Pad software (version-3).

RESULTS
Estimation of hepatic biomarkers
Figures 1 and 2 point out the intergroup differences in serum hepatic biochemical parameters like ALP, AST, ALT, TB, DB, and TPC levels. The present data revealed that the administration of 5-Fluorouracil produced a significant ($P<0.001$) elevation in serum ALP, AST, ALT, TB and DB levels while a significant reduction in TPC levels in 5-FU control group relative to the normal control group. In contrast, pre- and co-treatment with GA at the doses 30 and 100 mg/kg significantly ($P<0.01$ and 0.001, respectively) and dose-dependently ameliorated the alterations in the hepatic biomarkers (Figure 1 and Figure 2).

Histopathological studies of hepatic tissue
Histopathological investigation of hepatic tissues of the normal control group showed the presence of normal hepatocytes and central vein. Livers of 5-FU control group rats showed

marked reactive changes suggestive of hydropic degeneration (HD) of the hepatocytes as well as focal necrosis (FN) at the central vein zone with the disruption of the hepatic central vein (HCV). These histopathological alterations were found to be ameliorated dose-dependantly by the pre- and co-treatment with GA at doses 30 and 100 mg/kg showing moderate and marked improvement respectively in histopathological alterations in liver tissues (Figure 3).

DISCUSSION

Liver is the main detoxifying organ that after exposure to frequent and high doses of cancer chemotherapy results in hepatotoxicity.[31] Hepatotoxicity is the most common adverse effect of 5-FU as it is largely metabolized in the liver. The toxic metabolites produced by 5-FU tend to initiate hepatic injury exhibiting severe hepatotoxicity limiting its chemotherapeutic usefulness as an efficacious anticancer agent. The underlying mechanisms associated with 5-FU induced hepatotoxicity include increased apoptosis, oxidative stress and inflammatory reactions.[29] In our study, we estimated different hepatic biomarkers to confirm 5-FU induced hepatotoxicity. Serum transaminases such as AST, ALT and ALP have been considered as important indicators of hepatic damage[32]. ALT is an important cytosolic enzyme which is more specific for the liver. Enzyme AST is located in hepatic mitochondria, ALP is an hepatic enzyme whose activity is plasma increases as a result of obstruction or inflammation of the biliary tract.[33] Increase in their levels in the blood is majorly a result of leakage of these transaminases from the hepatocytes into the circulation, indicating liver damage or dysfunction.[32] 5-FU caused a severe increase in the levels of hepatic biomarkers i.e alkaline phosphatase (ALP), aspartate aminotransferase (AST) alanine aminotransferase (ALT), direct bilirubin and total bilirubin with decreased total protein content in comparison with normal control group showing severe hepatotoxicity. These results were found to be in agreement with the previous studies[29]. The reversal of transaminase levels to normal by GA in the present study exhibited its hepatoprotective activity by reducing hepatic injury and inflammation. Total and direct bilirubin are the indicators of the normal functioning of the liver, increase in these levels are found in hepatic disorders indicating severe hepatic damage leading to jaundice.[34] Reduction in the elevated levels of bilirubin by GA indicated its potent hepatoprotective activities and thus its usefulness in the treatment of various hepatic disorders caused by 5-FU.

Total protein content measures the total number of proteins present in body fluid. As the liver is involved in the synthesis of various proteins in the body, low protein content is considered as an important marker of hepatic damage and dysfunction in various hepatic disorders.[35] In our study, improvement in the total protein contents by GA indicated its hepatoprotective activity by reducing hepatic damage and dysfunction and it's potential in the treatment of 5-FU induced hepatotoxicity.

In the investigations of the potential protective drug against vital organ toxicities, the need for evaluating the drug efficacy on the histopathological measures of organ damage has been always emphasized.[35] Hence to support and confirm the results of biochemical estimations, histopathological studies of hepatic tissue were performed. In the current study, the histopathological changes seen in the hepatic tissues of the 5-FU control group in comparison with normal control group supported the concept of 5-FU induced hepatotoxicity and were concomitant with the significant alterations in hepatic biomarkers found in this study. The histopathological alterations included hydropic degeneration and necrosis of the hepatocytes with the hepatic central vein disruption that were similar to the previous findings[37]. The major

documented mechanisms of 5-FU include hepatic inflammation, apoptosis and oxidative stress. As GA has been documented to possess anti-inflammatory and antioxidant properties, these may be possible mechanisms of hepatoprotective activity of GA against 5-FU induced hepatotoxicity. GA has been already documented for its hepatoprotective activity against cyclophosphamide-induced hepatotoxicity by restoring hepatic antioxidant enzyme levels and reducing in micronuclei formation and DNA fragmentation which can also be added as a possible mechanism in this regard.[28]

CONCLUSION

Summarising the results of the investigation conclusion can be drawn that in the present study, the 5-FU exhibited severe hepatotoxicity confirmed by severe alterations in biochemical and histopathological parameters. GA was evaluated against 5-FU induced hepatotoxicity, which showed its protective role in attenuating the hepatotoxicity by ameliorating these alterations in a significantly and dose-dependentlyr. The chemoprotective potential of GA may be ascribed to its anti-inflammatory and antioxidant properties. Hence it was proved that pre- and co-administration of GA can overcome the 5-FU chemotherapy-induced toxicities which may help the cancer patients to maintain their well being during and after the chemotherapy thereby enhancing the life expectancy of cancer patients. The conclusions regarding optimum dose and probable mechanisms will also be useful for future studies. The conclusions of the study can form the basis for the design of suitable clinical studies.

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CONFLICT OF INTEREST: There is no conflict of interest

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Figures, figure legends and illustrations:

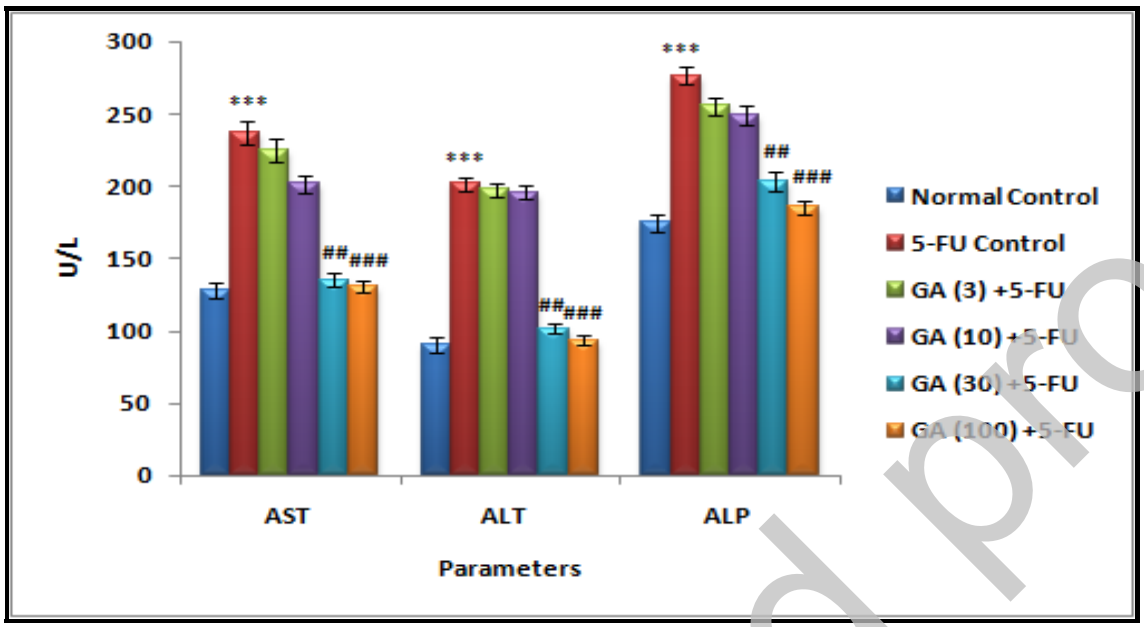

Figure 1: Effect of GA on serum levels of AST, ALT and ALP in 5-FU induced hepatotoxicity in rats

Results were expressed as mean ± SEM (n=6). Comparison between the groups was made by one-way analysis of variance (ANOVA) followed by Tukey's Kramer Multiple Comparison test $^{***}P<0.001$ *as compared to normal control.* $^{##}P<0.01$, $^{###}P<0.001$ *as compared to 5-FU induced control*

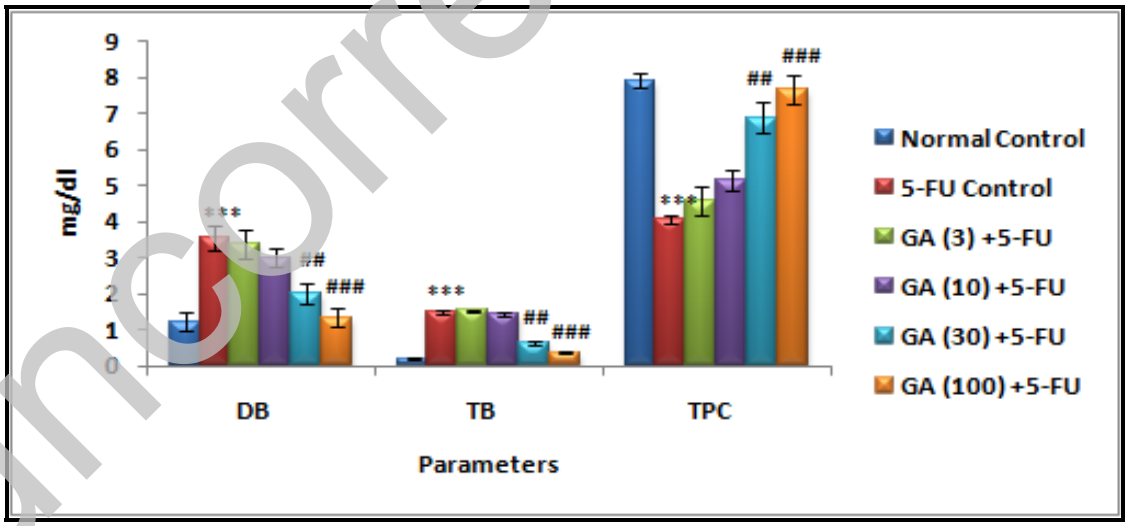

Figure 2: Effect of GA on serum levels of DB, TB and TPC in 5-FU induced hepatotoxicity in rats

Results were expressed as mean ± SEM (n=6). Comparison between the groups was made by one-way analysis of variance (ANOVA) followed by Tukey's Kramer Multiple Comparison test

$^{***}P<0.001$ as compared to normal control. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ as compared to 5-FU induced control

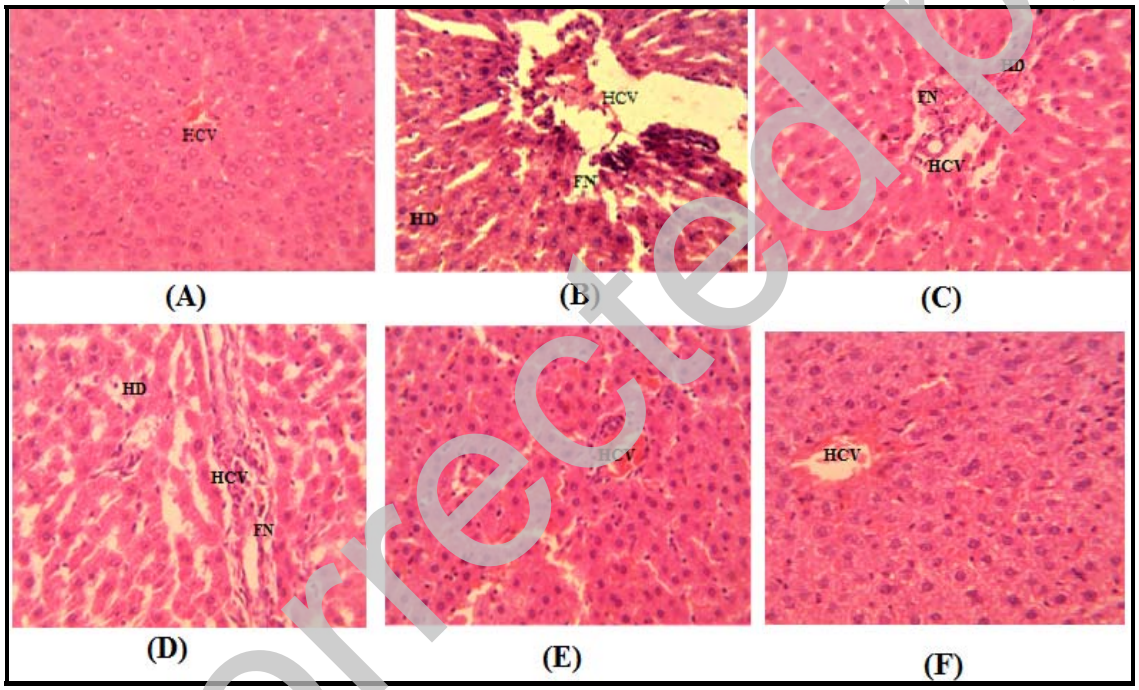

Figure 3: Effect of GA on 5-FU induced histopathological changes in the hepatic tissue
Representative photomicrographs (H & E stain) of liver sections of:
(A) Normal control rat showing normal liver architecture with normal hepatic central vein (HCV) and hepatocytes (B) 5-FU control rat showing marked reactive changes suggestive of hydropic degeneration (HD) of the hepatocytes as well as focal necrosis (FN) at central vein zone with the disruption of the hepatic central vein (HCV) (C) GA (3) + 5-FU and (D) GA (10) + 5-FU group rat showing similar reactive changes as 5-FU control with no improvement (E) GA (30) + 5-FU showing a moderate reduction in the reactive changes caused by 5-FU (F) GA (100) + 5-FU showing marked, amelioration of histological alterations caused by 5-FU Photographs taken through under high magnification power 45X using a trinocular microscope (Olympus CX-21-TR) with a camera (Magnuspro eyepiece camera software)