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PHARMACOLOGICAL EVALUATION GERMANIUM (IV)-HESPERIDIN COMPLEX FOR HEPATOCELLULAR CARCINOMA ON RATS

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ABSTRACT

Purpose: To investigate the anti-carcinogenic activity of Germanium-Hesperidin (Ge-Hp) complex in hepatocellular carcinoma (HCC) induced in rats. **Methods:** Female albino rats were divided into 4 groups of 10 animals each. Group 1 was the normal control. Group 2 were given diethylnitrosamine 200mg/kg in 0.1% carboxymethylcellulose (CMC)/ day for 4 weeks. Group 2 and Group 3 received test drug (Ge-Hp) and doxorubicin (standard drug) after induction of HCC for one month. All the animals were sacrificed at the end of treatment protocol and their biological samples were used to determine ALT, ALP, LDH, total bilirubin and alphafetoprotein. Weights of the liver and liver tumors were measured. Histopathology was performed on the rats of each group. **Results:** The gross tumor characteristics showed reduction in macro and micronodules in Ge-Hp treated compared to DENA treated control group. The serum levels of AFP, ALP, SGOT and SGPT in Ge-Hp Vs standard drug (doxorubicin) are (9.95±0.5 vs 7.23±0.8), (299.8±3.1 vs 309.3±3.1), (79.83 ± 4.93 vs 80.17 ± 2.98), and (61.83 ±2.798 vs58.83 ±2.372). **Conclusion:** Ge-Hp complex decreased the elevated levels of AFP, ALP, LDH, SGOT and SGPT in hepatocellular cancer on rats. All these parameters suggest that the complex Ge-Hp prove to have significant and potential against DENA induced cancerous lesions.

KEYWORDS: Hepatocellular carcinoma, germanium, hesperidin, DENA, doxorubicin, alphafetoprotein.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common liver malignancy and is one of the leading causes of cancer-related death worldwide. 841080 new cases and 781631 deaths were recorded under HCC according to GLOBOCAN 2018.[1] The most important risk factor for the development of HCC remains to be the cirrhosis of liver, regardless of etiology that can occur at the consequence of viral hepatitis, NAFLD and genetic disease. The current standard of care for HCC is surgery, liver transplantation, ablation, arterial directed therapy, radiation therapy, chemotherapy and mainly targeted systemic therapy by sorafenib, lenvatinib, atezolizumab and bevacizumab and, immune therapy.[2]

Metal complexes contain a variety of structural and electronic features that can be exploited in drug design.The urgency to overcome the biophysical and biomedical drawbacks of current chemotherapeutic treatments led the scientists to consider flavonoid metal complexes as viable options in cancer therapy. Both *in vitro* and *in vivo* studies report that flavonoids and their metal ion complexes exert pleiotropic effects on tumor promotion and progression.

Germanium (Ge) is a lustrous, hard, grayish-white metalloid in the carbon group, chemically similar to its group neighbors tin and silicon. Ge is not thought to be an essential element for any living organism. Some complex organic germanium compounds are being investigated as possible pharmaceuticals. Hesperidin (Hp) is a flavonoid that is abundantly found in citrus fruits. Hp which consists of a glycone (hesperidin) attached to a disaccharide unit composed of rhamnose and glucose is found mainly in the peel of orange and lemon and is usually isolated from Citrus aurantium, C. sinensis (Rutaceae). Hp has been shown to inhibit skin carcinogenesis. Although citrus fruits and juices are widely consumed in the world, little information has been published on flavanone bioavailability in humans. Hesperidin itself is absorbed from the intestine intact as a glycoside. Its aglycone hesperidin appears in plasma 3 h after ingestion, reaching a peak between 5 and 7 h. The circulating forms of hesperidin are glucuronides (87%) and sulphoglucuronides (13%). Several investigators have examined Hesperidin antioxidant activity and radical scavenging properties using a variety of assay systems.[3,4] Jovanovic et al.[5] reports that Hp reduces superoxide ions in electron transfer plus concerted proton transfer reaction in vitro. Further, Hp was found to be effective in protecting liposomes from UV-irradiation induced peroxidation, probably by scavenging the oxygen free radicals generated by UV irradiation.[6] Numerous studies confirmed a potent bioactivity of Hp, such as effects on the vascular system (reduces capillary permeability)[7],

anti-inflammatory effects, antioxidant effect, action on enzymes, antimicrobial activity (antibacterial, antifungal, antiviral), anticarcinogenic activity[8], cell aggregation inhibition, antiallergic effects, UV protecting activity, radioprotection, and so on. Hp and naringenin increased cytotoxicity of doxorubicin on MCF-7 cells and HeLa cells.

Our study aims to evaluate the chemotherapeutic property of a novel metal complex of germanium-hesperidin in DEN-induced hepatocellular carcinoma in rats.

MATERIALS AND METHODS

2.1. *Animals and Chemicals*

Laboratory bred Albino rats of female sex, weighing between 110-160gms were housed. They were kept in laboratory conditions at 24±2C, relatively humidity 60±5%, 12:12 photoperiodic condition, in well ventilated animal house in polypropylene cages with paddy husk as bedding and access to food and water ad libitum. During the experiment period the animals were fed standard feeding pellets procured from Hindustan Lever, India. The experimental protocol was approved by the Institutional Animal Ethical Committee (IACE/RCP) having registration number as1458/PO/E/11/CPCSEA. All conditions were in accordance to CPCSEA norms and "WHO" guidelines for the care and use of animals in scientific research.

2.2. *Preparations of test drug suspension*:

Hesperidin was moderately solubilized in water, the test sample was prepared as an aqueous suspension using 0.1% aqueous CMC & were given as oral suspensions. The suspension was evaluated using IR spectra, UV Visible spectra and DSC.

2.3. Toxicity Study

The subacute toxicity study of Group 3 (DEN + Test drug) was conducted for 1 day according to OECD guidelines with maximum dose.[9] For that we have fasted six animals for 4hours and administered orally. According to limit test maximum dose of 5000mg/kg were given. Single animals are dosed in sequence usually at 48 h intervals. It was seen that three rats survived. However, the time intervals between dosing are determined by the onset, duration, and severity of toxic signs. Treatment of an animal at the next dose should be delayed until one is confident of survival of the previously dosed animal. The time interval may be adjusted as appropriate, e.g., in case of inconclusive response. The test is simpler to implement when a single time interval is used for making sequential dosing decisions.

Nevertheless, it is not necessary to recalculate dosing or likelihood-ratios if the time interval changes midtest. For selecting the starting dose, all available information, including information on structurally related substances and results of any other toxicity tests on the test material, should be used to approximate the LD50 as well as the slope of the dose-response curve. However the mortality rates were not seen and hence the maximum dose was selected for treatment purposes.

2.4. *Induction of hepatic carcinoma*

After weighing rats of weights around 110-160 gms. They were segregated into groups and then they were given diethylnitrosamine. DEN was purchased from Sigma Aldrich. For induction the intraperitoneal site was chosen for administration as it had less complications and lesser mortality rate during procedure. All groups were given 200mg/kg body weight calculated intraperitoneally.[10]

At the end of the treatment protocol (2weeks), animals were anesthetized with ether and blood samples were drawn from the orbital venous plexus. Serum was separated by centrifugation for 5 min at 1500g and stored at -20 degrees until analysis. This was then used to determine ALT, ALP, LDH and total bilirubin. All animals were sacrificed by decapitation and their livers were rapidly excised, weighed, washed with saline, blotted with a piece of filter paper and homogenised in normal saline or 6% perchloric acid as indicated in the procedures of measurement of each parameter. Preparation of histopathological slides was also created.

2.5. *Pre-clinical screening procedures*

Rats were randomly divided in four groups of 8 subjects each, subjected to 24hrs of fasting and orally treated as follows.

Group 1- Normal control (administered with vehicle 0.1% CMC i.e 0.5ml/100ml) 4 weeks

Group 2- DENA control (administration with 200mg/kg + 0.1% CMC) 4 weeks

Group 3- DENA + Test drug complex (Germanium IV hesperidin complex=200mg/kg) (2 weeks + 2weeks)

Group 4 –DENA + Standard drug treated (Doxorubicin) (2weeks+2weeks) (20mg/kg)

Table 1: Chemicals.

Chemical	Source
Hesperidin	Sigma Aldrich Corporations, USA
Germanium	Sigma Aldrich Corporations, USA
Diethylnitrosamine (DENA)	Sigma Aldrich Corporations, USA
CMC	Bengal Lab Pvt. Ltd
Distil water	Bengal Lab Pvt. Ltd
Ethanol	Bengal Lab Pvt Ltd
Formaldehyde 95%	MERCK Industries
NaCl 0.9 %	Bengal Lab Pvt Ltd
Diethyl ether	MERCK Industries

Table 2: Instruments.

Item	Source
Analytical Balance	ML-2004 Pioneer Industries
UV Visible spectrophotometer	UV-1800 Shimadzu, Japan
Mechanical Stirrer	Remi equipment's, Mumbai, India
Micro Centrifuge (SPINWIN)	Lab equipment's and chemicals Kolkata
FT-IR	Bruker, Germany

2.6. *Statistical Analysis*

GraphPad Prism software version 7.03 for Windows had been used for statistical analysis. Data are furnished as mean + s.e.m. Early death after challenging the animals with the inducing agent was an exclusion criterion.

RESULTS

The IR spectra of Ge-Hp and their complex is shown in **Fig. 1**. The IR spectra of the complex are similar to that of hesperidin. This might be attributed to the high quantity of Hp in the complex samples. And in the IR spectrum of their complex and mixture samples, some small characteristic absorption peaks of Hp between 601.04 and 1664.63 cm^{-1} were almost masked by Ge. In IR analysis, new significant peaks were observed in the complex samples compared with that of Hp. These results indicated that there were some physical interactions between Hp and Ge during the formation of their complex samples. The UV spectra of Ge, Hp and their complex and mixture samples have been done and shown in **Fig. 2**. UV analysis and their characteristic absorption wavelengths were present at 373nm. Furthermore, DSC curves of Ge, Hp and their complex and mixture samples had been observed where two endothermal peaks were obtained from the DSC curves of Hp in **Fig. 3**. The first endothermal peak with onset at 103ºC could be due to the removal of crystal water from Hp molecules. The second endothermal peak with onset temperature at 252ºC was due to the melting of hesperidin.

Three endothermal peaks were obtained in the DSC curve of the mixture sample and this might be attributed to the effect of Ge and hp. But there was only one peak of in the DSC curve complex and similar to that of Ge. These indicated the above endothermal peaks of Hp disappeared in the complex samples. According to the above results we can conclude that Hp has been completely dispersed in Ge but there must be some van der wall force occurring in this interaction. The gross tumor characteristics and histopathology are represented in **Fig 4** and **Fig 5**. The results of the tumor characteristics, liver function and other blood biomarkers were found to be favourable after Ge-Hp treatment as depicted in **Tables 3,4 and 5**.

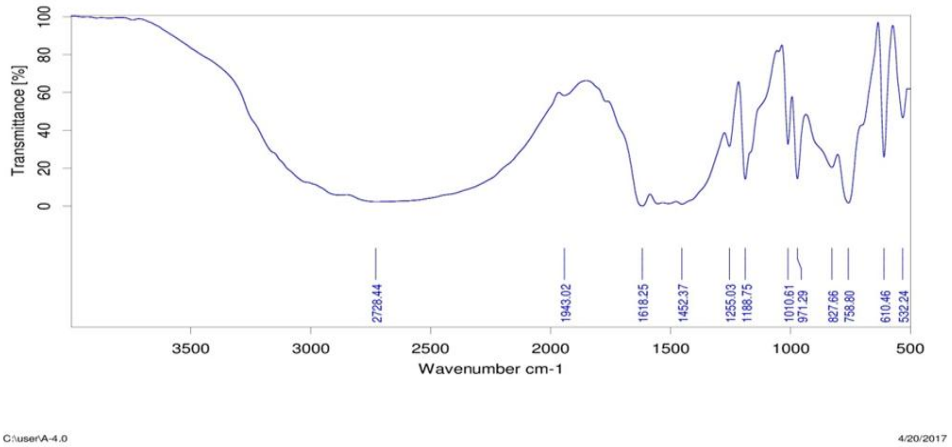

Fig 1: IR spectra analysis of Ge-Hp complex.

Table : UV reading of Hesperidin Germanium Complex at 373nm Wavelength

Concentration of the conjugate	Absorbance at 373nm
DMSO(10mcg/ml)	0.372
DMSO(30mcg/ml)	1.15
DMSO(60mcg/ml)	1.95

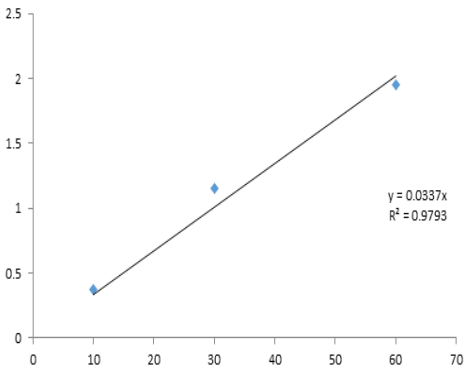

Fig 2: UV analysis and absorption curve of Ge-Hp complex.

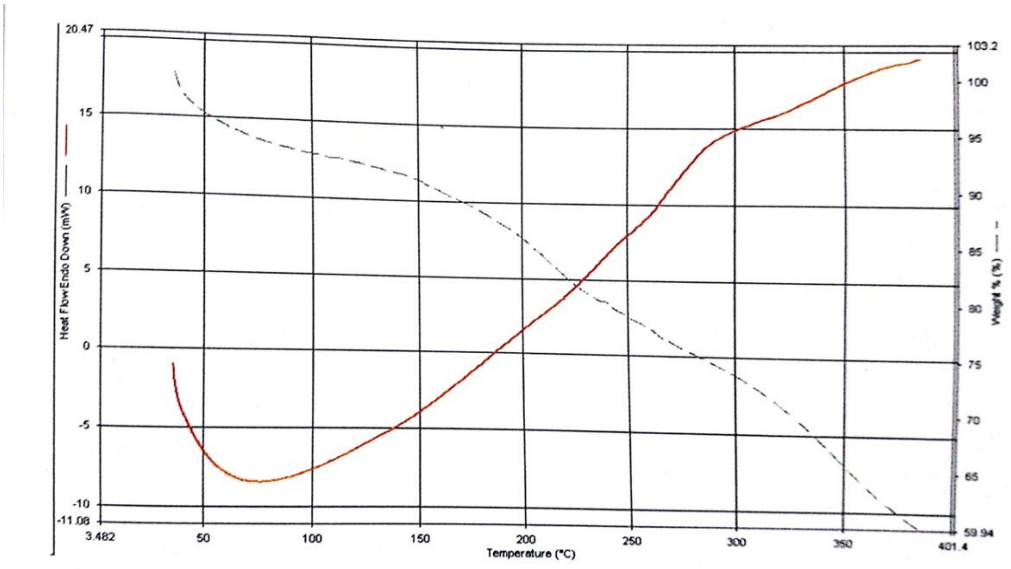

Fig 3: The DSC curve is represented for Ge-Hp complex.

a

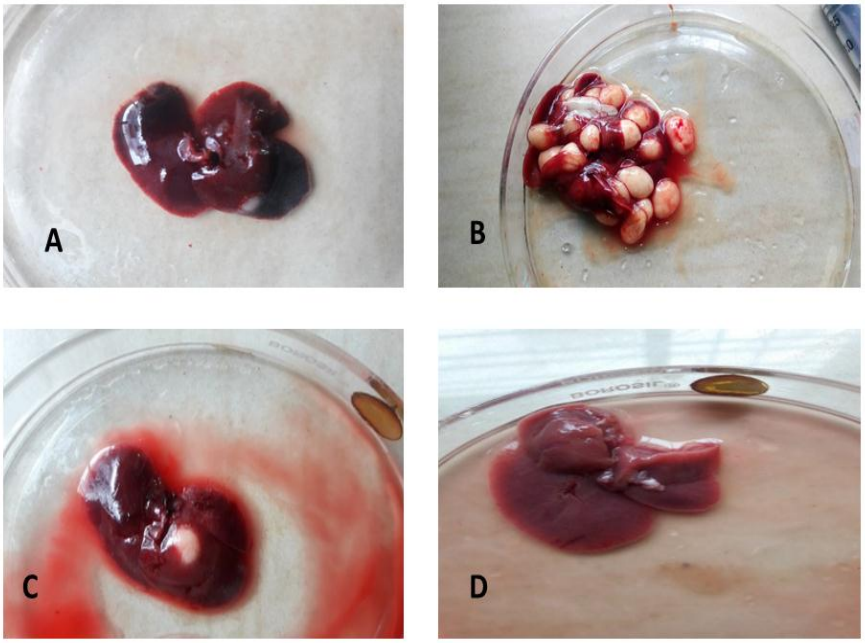

Fig 4: Gross liver characteristics. A: Normal liver; B: DENA control liver; C: DENA+ Ge-Hp treated liver; D: DENA+ Doxorubicin treated liver.

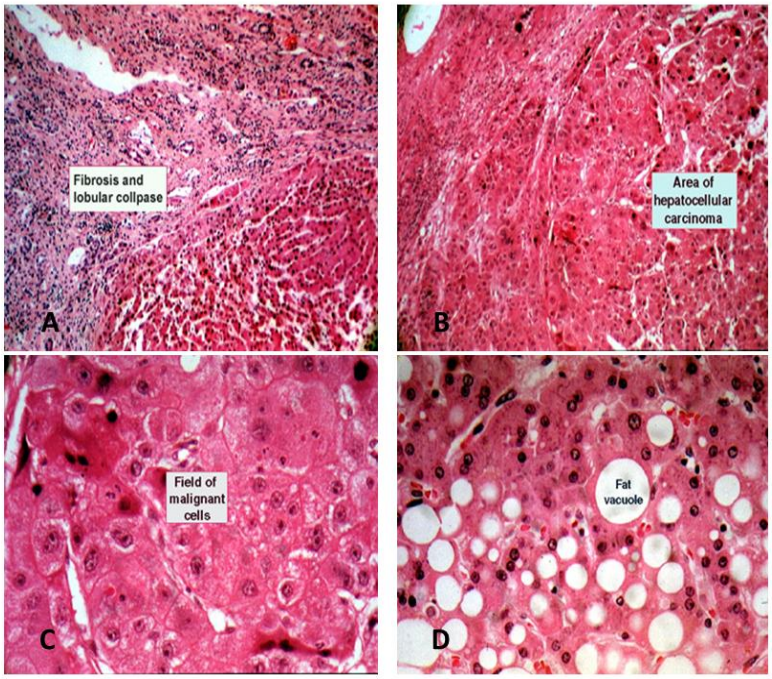

Fig 5: Histopathology of DENA induced HCC.

A: Under 10X zoom we observe Hepatocellular carcinoma (HCC) in its histologic identification.

B: Under 10X observational study Portal tracts are not seen either in hepatocellular adenomas or HCC but large caliber veins and arteries are often seen.

C: Under 40X zoom One of the hallmark histopathologic features of HCC is the presence of endothelial cells (arrow) lining the sinusoids that surround enlarged hepatocellular plates/cords. Malignant cells are seen here.

D: Presence of fat in well-differentiated tumor.

Table 3: Gross tumor characteristics in different groups.

Category	Normal Control	DEN Control	DEN + Ge-HP	DEN+ STD. Drug
1) Liver weight	5.683 ± 0.2	11.33 ± 0.5	7.600 ± 0.2	6.483 ± 0.2
2)Liver texture	Chestnut Brown	Dark Red	Pale	Blemish Pale
3)Macronodule	NIL	8.83 ± 0.6	2.0 ± 0.3	1.33 ±0.2
4)Micronodule	NIL	7.66 ± 0.6	2.66 ±0.2	1.66 ±0.2

Table 4: Liver function tests.

Treatment groups	Bilirubin Total	SGOT	SGPT	TC	Lymphocyte
Normal Control	0.36 ± 0.03	57.50± 2.07	30.83 ±2.574	2883 ± 212.0	26.83 ± 0.6
DEN Control	14.42 ± 0.34	20.3 ± 3.56	76.00 ±3.055	5083 ± 454.2	62.83 ± 4.5
DEN+ Ge-HP	0.98 ± 0.06	79.83 ± 4.93	61.83 ±2.798	3183 ± 137.0	30.83 ± 0.6
DEN +STD.DRUG	0.61 ± 0.03	80.17 ± 2.98	58.83 ±2.372	2708 ± 240.3	28.17 ± 1.5

Table 5: Blood biomarkers in different treatment groups.

Treatment groups	AFP	ALP	LDH
Normal Control	4.13± 0.2	165.3±1.1	7.866 ±3.2
DEN Control	13.8±0.5	373.8±5.4	35.29 ±4.4
DEN + Ge-HP	9.95±0.5	299.8±3.1	14.55± 5.9
DEN+ STD.DRUG	7.23±0.8	309.3±3.1	12.44±5.07

DISCUSSION

The study fulfils the aim to report the novel Ge-Hp complex as a successful anti-cancer agent against hepatocellular cancer in rats. The results are similar to another study where six-

coordinate Ge (IV)-diketonate was found to have anti-cancer properties.[11] Organic germanium can be preventive of intestinal cancer in animal models, as Jao et al., 1990.[12]

Hp suppressed expression of Rassf7, Nrf2, PARP and NF-κB in a dose dependent manner with a maximum inhibition at the level of 300 mg/kg body weight hesperidin. In conclusion, oral administration of hesperidin protected mice against chemical carcinogenesis by increasing antioxidant status, reducing DMBA+TPA induced lipid peroxidation and inflammatory response, and repressing of Rassf7, Nrf2, PARP, and NF-κB levels.[13]

The results of MTT and crystal violet assay evaluating cytotoxic effect of Hsp-AuNPs (Hesperidin gold nanoparticles) on human breast cancer cell line (MDA-MB-231) revealed significant decrease in proliferation and inhibition of growth of the treated cells when compared with normal human breast epithelial cell line (HBL-100). The production of the pro-inflammatory cytokines (IL-1β, IL-6 and TNF) in bonemarrow–derived macrophage cells treated with Hsp-AuNPs was significantly inhibited.[14]

There were several supportive evidences of Hp and Ge bearing anti-cancer properties individually but in the present study we have assessed the cumulative effect of both the test drugs. It can be stated that this study of pharmacological evaluation is reliable pre-clinical models for hepatocellular cancer study. The reports also suggest that hesperidin proves to be hepatoprotective and playing a very crucial role in minimising the ill effects of hepatocellular carcinoma. U.V studies indicated complex-formation between HP and some metal ions in DMSO. It can also be stated that none of the complexes produced aberrant properties in the solution mixtures. IR studies were very significant as the metal complex seemed to be increasing the affinity of intercalative binding to DNA that suggests DNA base pairs where HP is encouraged to form complex with metal ions. Hp was found to be having less inhibitory action against alpha-amylase and glucosidase enzymes and complexation did not produce significant improvements in HP behaviour.

Even though the test drug was not found to be superior to the standard drug, However, these antioxidants may have lesser toxicity when compare to the traditional chemotherapeutic agents. Further studies are needed to optimise a safer dose of Ge-Hp that is superior to the standard drug and comparative analysis of the safety and toxicity profile are a required in a larger sample size.

CONCLUSION

Hes-Ge complex decreased the elevated levels of AFP, ALP, LDH, SGOT and SGPT levels activity in hepatocellular cancer in rats. All these parameters suggest that the complex HES-GE prove to be significant and potential against DENA induced cancerous lesions.

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DECLARATIONS

CONFLICT OF INTEREST: The authors declare no competing interests.

ETHICS APPROVAL: The experimental protocol was approved by the Institutional Animal Ethical Committee (IACE/RCP) having registration number as1458/PO/E/11/CPCSEA.

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REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: a cancer journal for clinicians*, 2018 Sep 12; doi: 10.3322/caac.21492.
2. Balogh, J., Victor, D., 3rd, Asham, E. H., Burroughs, S. G., Boktour, M., Saharia, A., Li, X., Ghobrial, R. M., & Monsour, H. P., Jr (2016). Hepatocellular carcinoma: a review. *Journal of hepatocellular carcinoma*, *3*: 41–53.
3. Md. Nur Alam *, Nusrat Jahan Bristi Review on in vivo and in vitro methods evaluation of antioxidant activity Saudi Pharmaceutical Journal, 2013; 21: 143–152.
4. Prawez Alam*, Aftab Alam Quantitative estimation of hesperidin by HPTLC in different varieties of citrus peels Asian Pacific Journal of Tropical Biomedicine.
5. Rachmat Mauludin Physicochemical Properties of Hesperidin Nanocrystal International Journal of Pharmacy and Pharmaceutical Sciences, 2013; 5(3).
6. Martijn WH Leenders Mouse models in liver cancer research: A review of current literature World Journal of Gastroenterology.

7. Edward J. Victoria and Albert A. Barber, Peroxidation of Microsomal Membrane Protein-Lipid Complexes Research sponsored by AEC grant AT (11-1), 34 Pro. 49 and by American Cancer Society grant, 422.
8. Ayse Arslan1, Halit Demir Evaluation of Lipid Peroxidation and Some Antioxidant Activities in Patients with Primary and Metastatic Liver Cancer Journal of Cancer Therapy, 2014; 5: 192-197. Published Online February (http://www.scirp.org/journal/jct)
9. OECD (2001), (OECD GUIDELINE FOR TESTING OF CHEMICALS), https://doi.org/10.1787/9789264071001-e (Accessed on 17th July 2020).
10. Krishnan GS, Rajagopal V, Antony Joseph SR, Sebastian D, Savarimuthu I, Selvaraj KRN, Thobias AF. In vitro, In silico and In vivo Antitumor Activity of Crude Methanolic Extract of Tetilla dactyloidea (Carter, 1869) on DEN Induced HCC in a Rat Model. Biomed Pharmacother, 2017 Nov; 95: 795-807 Epub 2017 Sep 10. PMID: 28892791.
11. Randall T. Mertens, Sean Parkin, Samuel G. Awuah, Exploring six-coordinate germanium(IV)-diketonate complexes as anticancer agents, Inorganica Chimica Acta, 2020; 503: 119375, ISSN 0020-1693.
12. Jao, SW., Lee, W. & Ho, YS. Effect of germanium on 1, 2-dimethylhydrazine-induced intestinal cancer in rats. *Dis Colon Rectum,* 1990; 33: 99–104.
13. Mathipi Vabeiryureilai, Khawlhring Lalrinzuali, Ganesh Chandra Jagetia. Chemopreventive effect of hesperidin, a citrus bioflavonoid in two stage skin carcinogenesis in Swiss albino mice, Heliyon, 5(10): e02521, ISSN 2405-8440.
14. Sulaiman, G.M., Waheeb, H.M., Jabir, M.S. et al. Hesperidin Loaded on Gold Nanoparticles as a Drug Delivery System for a Successful Biocompatible, Anti-Cancer, Anti-Inflammatory and Phagocytosis Inducer Model. Sci Rep, 2020; 10: 9362.