
1PATHOLOGICAL EVALUATION OF SOME TRADITIONALLY USED MATERIALS IN HUMAN AND VETERINARY PRACTICE

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ABSTRACT

Recently, there are growing interests especially in the developed countries in using some materials from the folk medicine for treatment of some diseases that are not curable by current medications like cancer and hepatitis C virus. This article is focusing on pathological evaluation of three of these materials turmeric, camel's milk and curcumin. Camel's milk and turmeric were tested as hepatoprotective against Carbon tetrachloride toxicity in albino rats and they partially protect against that toxicity. In another experiment curcumin and Meriva (curcumin phospholipid complex) were evaluated as anticancer agents. *In vitro*, curcumin induced cell death in dose-dependent manner. *In vivo*, both curcumin and Meriva have failed anticancer agents against canine glioblastoma in nude mice.

INTRODUCTION

Nowadays, medicinal plants have gained much public interest worldwide in treatment of human diseases as they more natural and less toxic than the currently used chemicals drugs. In Egypt, many people have started to use camel's milk for treatment of hepatitis C virus (HCV) and this is not associated with any scientific proof. Egypt

holds a unique position in the epidemiology of hepatitis and liver cancer. Egypt reports the highest prevalence of HCV worldwide (1).

Between the period of 1996 to 2001, brain and other CNS cancers were accounted for 4.8% of all Egyptian cancer (9). It has been estimated that more than two third of human cancers could be prevented by modification of life style including die-

tary modification (27). Research over the last decade has shown that several micronutrients in fruits and vegetables reduce cancer. The active components of dietary phytochemicals that most often appear to be protective against cancer are curcumin, genistein, resveratrol, diallyl sulfide, S-allyl cysteine, allicin, lycopene, capsaicin, diosgenin, 6-gingerol, ellagic acid, ursolic acid, silymarin (2).

Curcumin (diferuloylmethan), a yellow colored polyphenol, is one of the most studied natural compounds which have been used as a feed additive for centuries. Curcumin is an active principle of the perennial herb *Curcuma longa* (commonly known as turmeric). It has a wide range of beneficial properties, including anti-inflammatory, antioxidant, chemopreventive and chemotherapeutic activity and inhibits cell signaling pathways at multiple levels (10, 24). Studies of curcumin in animals have confirmed a lack of significant toxicity since an early report in which doses up to 5 g/kg were administered orally to Sprague-Dawley rats, but exhibit poor bioavailability (3, 29).

In a rat experiment, Conjugation of curcumin with phosphatidylcholine (Meriva) has increased the plasma levels and liver levels of curcumin compared to unformulated

curcumin. In contrast, curcumin concentrations in the gastrointestinal mucosa after ingestion of Meriva were somewhat lower than those observed after administration of unformulated curcumin. Similar observations were made for curcumin metabolites as for parent compound (17).

This article aimed to evaluate the use of Curcumin formulated with phosphatidylcholine (Meriva) and unformulated curcumin in the treatment of canine glioblastoma and figure out if this conjugation improved the effect of curcumin or not. Also, Study the effects of camel's milk and turmeric in the treatment of experimentally induced liver damage

MATERIALS AND METHODS

CHEMICALS

Curcumin was purchased from sigma chemical Co. (St. Louis, Mo). Curcumin was prepared as a stock solution of 40mM and stored at -20° C until use. Curcumin phospholipid complex (Meriva) was provided by Indena SPA (Milan, Italy). The preparation of Meriva made using EpiKuron 130 P, a de-oiled, powdered soybean lecithin enriched with 30% phosphatidylcholine. Meriva contained 16.89% curcuminoids, of which 93.82% was curcumin (17). Dulbecco's modified Eagle's medium (DMEM), HANK's balanced salt solution and fetal bovine

serum (FBS) were purchased from Invitrogen Inc (Carlsbad, CA, USA). *Anti-caspase 3* antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Camel's milk was obtained from bedouins at El-Arish governorate. *Curcuma longa* (Turmeric) powder was purchased from Egyptian market. Carbon tetrachloride was purchased from Al-Pharana laboratory chemicals.

CELL LINES

A D-GBM canine glioblastoma cell line was isolated by Dr. Stoica from 8-years-old male Boxer dog that was diagnosed with GBM grad IV (25). The cell line was maintained in DMEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 ug/ml streptomycin). The cells were grown at 37°C in a humidified incubator containing 5% CO₂ in air. Cells were passage biweekly and used for experiments when it is in the exponential growth phase.

CELL VIABILITY ASSAY

The antiproliferative effect of curcumin on D-GBM tumor cells was determined by using 96[®] AQueous one solution cell proliferation colorimetric assay kit (Promega, Madison, WI). The solution reagent contains a tetrazolium compound [3-

(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent phenazine ethosulfate (PES). The Cell Titer 96[®] AQueous assay uses phenazine methosulfate (PMS) as the electron coupling reagent. PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. Briefly, the cells were trypsinized and washed then resuspended in DMEM medium and seeded into 96-well flat-bottomed plate (1×10⁴ cells/well) in 6 replicate. Curcumin was added to each well in various concentrations (0-40µM). Cells were maintained in a humidified 5 % CO₂ and 95% air incubator at 37°C for 24 hrs or 48 hrs. Following washing of cells, 100 µl of fresh DMEM and 20 µl of the Cell Titer 96[®] AQueous One Solution were added per well and incubated at 37 °C for 1 h 30 min. All wells were sequentially read with a Biotek Synergy 4 plate reader (Biotek Instruments Inc., Winooski, VT, USA) using an absorbance wavelength of 490nm. Mean background values were obtained by scanning blank wells. Results were expressed as mean optical density (O.D.) corrected to background.

D-GBM CELL TUMOR XENOGRAFT IMMUNOHISTOCHEMISTRY (IHC)

Five-micron (5 μ m) paraffin-embedded sections were prepared from formalin fix tissues. The sections were deparaffinized in xylene and rehydrated with gradient alcohols. Antigen retrieval was performed by placing sections in citrate buffer (pH 6.0) or a decloaker pressure cooker for 15 min at 120° per 18 psi. Following cool-down, potential non-specific binding sites were blocked with 5% normal goat or rabbit serum in phosphate buffered saline (PBS). The sections were then incubated with anti-caspase 3 (1:200). After three 5 min washes in PBS, the sections were incubated with specific biotin-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA). A Vector-ABC streptavidin-peroxidase kit with a benzidine substrate was used for color development.

Counter-staining was done with diluted hematoxinilin. Sections that were not incubated with primary antibody served as negative control.

HEPATOTOXIC STUDY

In order to study the hepatoprotective effects of camel's milk and turmeric, a total number of 30 albino rats (2 months old) obtained from laboratory animal facilities at Helwan governorate, were housed in stainless steel wire-bottomed cages. Rats were given a balanced powder diet. Rats were orally administrated Ccl4 (4mg/kg) by intragastric tube. The animals were randomly divided into six groups (n=5) (see table 1). At the end of the study, rats were anesthetized with ethyl ether and sacrificed followed by complete necropsy, then the internal organs were immediately fixed in 10% neutral buffer formalin for histopathology.

Group	Treatment	Animals #
1	Control normal untreated	5
2	Control normal feed on camel's milk	5
3	Control normal feed on diet containing 2% turmeric	5
4	Control untreated and administrated Ccl4 (4mg/kg)	5
5	administrated Ccl4 (4mg/kg) then treated with camel's milk for 2 weeks	5
6	administrated Ccl4 (4mg/kg) then treated with turmeric for 2 weeks	5

STATISTICAL ANALYSIS

Data were presented as mean \pm SD. Comparison of data from two treatment groups was done using a Student t-test and the difference was considered significant at $p < 0.05$. Comparison of three treatment groups was done using one way ANOVA followed by Tukey multiple comparison test at $p < 0.05$.

RESULTS

CYTOTOXICITY OF CURCUMIN ON D-GBM

Prior to investigate the effect of Meriva and curcumin *in vivo*, we tested if curcumin has an anti-proliferative and cytotoxic effect on the D-GBM cells. Figure 1 showed that curcumin induced cytotoxicity in dose – dependent manner following 24hrs treatment. The cell viability started to decrease at 30 μ M following 24 hrs treatment. The cytotoxicity of curcumin was more severe at 35 μ M and 40 μ M by 48 hrs (Figure 2).

Effect of Curcumin and Meriva on Canine glioblastoma

Mice inoculated with canine glioblastoma cells were monitored everyday to detect the occurrence of the neurological signs. On the day 10th post inoculation the control and curcumin treated group began to show decreasing in the feed intake, decreased activity. On the day 14th the

mice of the control and curc-umin treated groups were emaciated and showed neurological signs as ataxia, hunched posture, head tilt, upon catching vigorously chalking themselves and rotating around their axis. The same signs appeared in the Meriva treated group on the day 16th.

Comparison between the control group and Meriva treated group in a repeated experiment was done, but with low number of inoculated tumor cells. The signs appeared in the two groups 20 days after the inoculation of the tumor cells. On necropsy, the mice had protruded skull indicative for tumor formation in the beneath of the skull (Fig. 3).

Microscopically, the neoplastic cells were expanding the meninges up to 4 times thickness than the normal and they invading deeper into the brain tissue (Fig. 4). The tumor cells were infiltrating through the brain ventricles (lateral and 3rd) and periventricular tissue (Fig. 5,6). The preexisting capillaries were surrounded by the neoplastic cells (Fig. 7). The neoplastic cells arranged as scattered nodules throughout the neuropil. The neoplastic cells were polygonal, with indistinct cell borders, moderate amount of eosinophilic cytoplasm, and round to oval nuclei with finely stippled chromatin. Mitotic figures reached up in some fields to 7 HPF (Fig. 8). There

were some inflammatory cells mainly macrophages and lymphocytes within the neoplasm. Within the tumor, there were scattered areas of necrotic cells characterized by highly eosinophilic cytoplasm and pyknotic nucleus (Fig. 9). In some areas the neoplastic cells infiltrating the bone tissue of the skull with the presence of many osteoclasts (Fig. 10). Immunohistochemically, There was no significant difference in the expression of caspase-3 and VEGF between the control, curcumin, and the Meriva groups (Data not shown).

Effect of turmeric and camel's milk Ccl4 toxicity

Following the administration of the Ccl4 and during the whole period of the treatment, the rats were apparently healthy and behaved normally. On necropsy, no gross lesions were detected except that liver and kidney of rats administrated Ccl4 without any treatment were congested.

Microscopically, the lesions of Ccl4 were mainly seen in the liver. The hepatocytes were diffusely swollen containing numerous, variable size, and well demarcated cytoplasmic vacuoles that pushed the nucleus to the periphery of the cells (degeneration) (Fig. 11&12). Portal vein, hepatic vein, and hepatic sinusoids were markedly congested (Fig. 13). Multifocally and randomly disrupting the hepatic architecture, there were small areas of coagulative necrosis characterized by round hyper-

eosinophilic hepatocytes that containing a pyknotic, karyorrhetic, or karyolytic nucleus. Multifocally expanding the portal areas, there were a little amount of fibrous connective tissue which was infiltrated with mononuclear cells mostly lymphocytes and plasma cells. The bile duct was mildly hyperplastic.

The extrahepatic lesions were mainly confined to the renal tissue. Renal tubules were lined with degenerative epithelium characterized by cytoplasmic vacuolation, pyknotic nucleus, and sloughing epithelial cells forming cellular casts within the tubular lumen. Mild sub-acute interstitial nephritis was evident by lymphocytic infiltration and congestion of the blood vessels. Spleen was slightly congested.

Rats treated with camel's milk and 2 % *Curcuma longa* for 2 weeks had mild hepatic degeneration. Most of hepatic tissue was normal except multifocal areas contained small, fine vacuoles with indistinct borders. The congestion and portal area of fibrosis was very mild. No inflammatory cells or necrotic cells were detected (Fig. 14). The renal tissue showed mild congestion.

DISCUSSION

The goal of this study was to evaluate the use of some natural agents that have gained much public interest in the last few years for treating diseases like cancer and hepatitis C virus in Egypt. We have chosen

curcumin, turmeric, and camel's milk as a three examples for these natural materials.

Curcumin, a well known dietary component from *Curcuma longa*, is an attractive candidate for drug development due to its ability to kill tumor cells and not normal cells. Our in-vitro experiments demonstrated that curcumin have induced cytotoxicity in canine glioblastoma (D-GBM) cell lines. Furthermore, *In vitro*, other studies indicated that curcumin has shown to behave as an antiproliferative and apoptotic agent in a wide variety of tumor cell lines and as an inhibitor of invasion, migration and metastasis. The mechanisms involve modulation of multiple cell signaling pathways of the tumor cells including cell proliferation pathway (cyclin D1, c-myc), cell survival pathway (Bcl-2, Bcl-xL, cFLIP, XIAP, c-IAP1), caspase activation pathway (caspase-8, 3, and 9), tumor suppressor pathway (p53, p21) death receptor pathway (DR4, DR5), mitochondrial pathways, and protein kinase pathway (JNK, Akt, and MAPK) (4,6,5,7,11,12,14,15,19,22,23,26).

Curcumin and Meriva were evaluated in xenograft animal model inoculated intracranially with canine glioblastoma cells. Some studies have demonstrated that curcumin

effectively reduced the oxidative damage and amyloid pathology in an Alzheimer transgenic mouse (13). In addition, curcumin was shown to have a neuroprotective effect in MCAO-induced cerebral ischemia in rats, which was mediated via its antioxidant activity (28). So, it is believed that curcumin is a promising agent for both prevention and treatment of glioma and other neurodegenerative diseases. In human, Glioblastoma multiforme (GBM) is the most common type of malignant brain tumor in adults and accounts for the majority of the 18,500 primary brain tumor cases diagnosed each year in the United States (20). Despite the various treatments including surgery, chemotherapy, and radiation therapy, GBM is one of the most lethal malignancies with a median survival of less than 2 years (16).

In our preliminary study, curcumin was not able to extend the survival of the nude mice. Meriva showed a weak effect as it increased the survival of the mice for 2 days only and the tumor was somewhat less invasive into the brain tissue. So we gave the Meriva a second chance with 2 advantage first lower number of the cell and we started the treatment 1 week before cell inoculation. We got surprised that Meriva didn't significantly increase the survival of the mice or decreased the invasion of the tumor. Our im-

munohistochemistry for cas-pase-3 to detect apoptosis were not showing any difference between the control, curcumin, and the Meriva. These results are in disagreement with that of Purkagatha et al. (21). They were able to block brain tumor formation in mice that had already received an intracerebral bolus of mouse melanoma cells (B16F10) by intracranial injection of a water soluble formula of curcumin through a cannula. This disagreement is coming from the difference route of curcumin administration. Their route is look like adding curcumin to the tumor cells in the *in vitro* study. Moreover this route is very difficult to be applicable in human or even in older rat with thick skull bone.

Egypt has suffered a big problem with hepatitis C Virus as huge number of Egyptians between the ages 30 to 60 years has been infected with this virus. These individuals are at greater risk for developing cirrhosis and/or hepatocellular carcinoma. Not only this leads directly to a drain on health resources, it also contributes to an indirect loss of the country's productivity. Considering the age group affected and the high rate of mortality, this disease removes people who are the most economically productive and who hold a great deal of social responsibility in terms of caring for children

and the elderly. Despite public health's longstanding knowledge of the problem, up till now there is no effective treatment for the virus or its associated lesions except liver transplant.

We evaluated the protective effect of turmeric and camel's milk against liver toxicity with Ccl4. Livers of rats treated with turmeric powder or camel's milk following Ccl4 administration showed only mild hepatic degeneration in comparison to severe hepatic degeneration in those rat administered Ccl4 without any treatment. That means turmeric and camel's milks are partially protecting liver against liver toxicity. Other studies have shown that turmeric extract treatment improve the hepatic parameters in the serum indicating the liver functions. That was not only against Ccl4 but also against doxorubicin toxicity (8, 18). Curcumin has expressed a protective effect against both acute and chronic hepatic toxicity on the clinical and histopathological level.

Overall, we can conclude that curcumin is a potent drug *in vitro*, but more work is needed to improve the absorption of that promising phytochemical as neither curcumin nor Meriva were not effective against canine glioblastoma. Moreover, Camel's milk and turmeric showed the ability to protect against the hepatotoxicity by Ccl4.

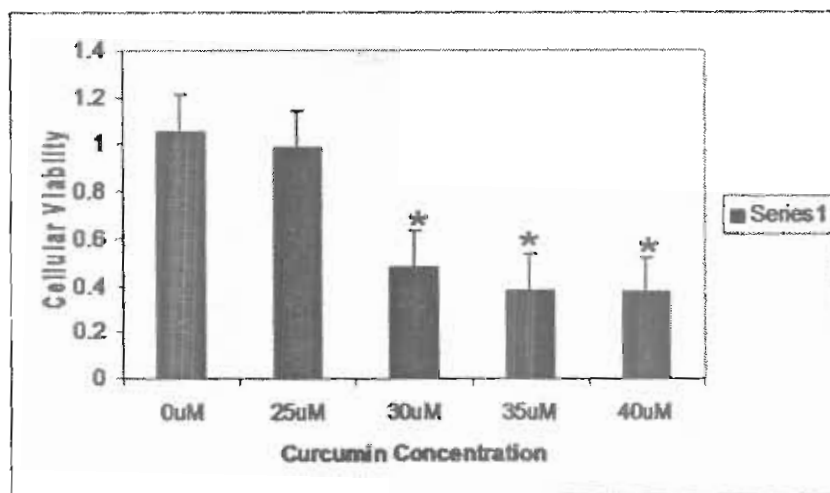


Fig. 1: The effect of different concentrations of curcumin on D-GBM cell following 24hrs. *indicates significant difference from control at $p < 0.05$.

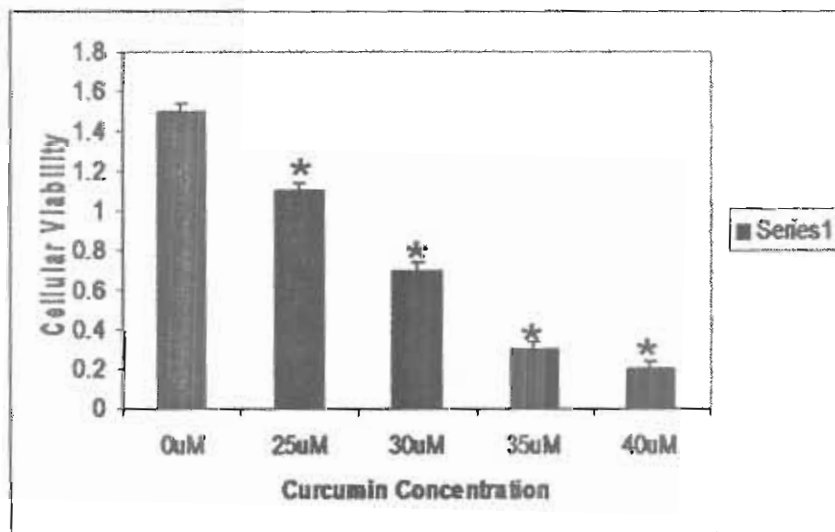


Fig. 2: The effect of different concentrations of curcumin on D-GBM cell following 48hrs. *indicates significant difference from control at $p < 0.05$.

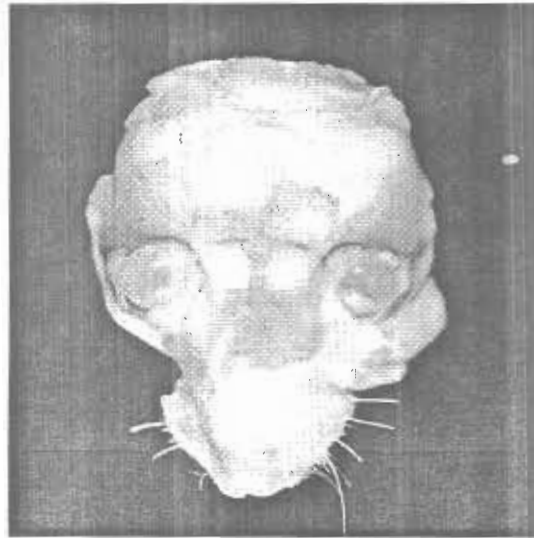


Fig. 3: Head of Athymic nude mice ,Meriva Group, inoculated with D-GBM showing protrusion of the tumor throughout the skull.

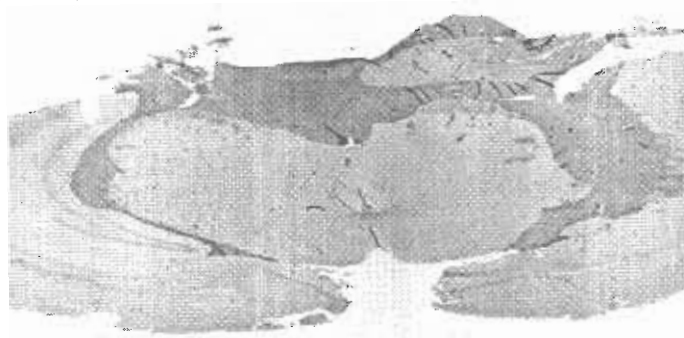


Fig. 4: Brain of mice from the control group, showing the neoplastic cells of D-GBM infiltrating the meninges and invading deep into the brain tissue (H&E X 10).



Fig. 5: Brain of mice from the control group, showing the neoplastic cells of D-GBM infiltrating within the brain ventricle and also in the periventricular tissue (H&E X40).

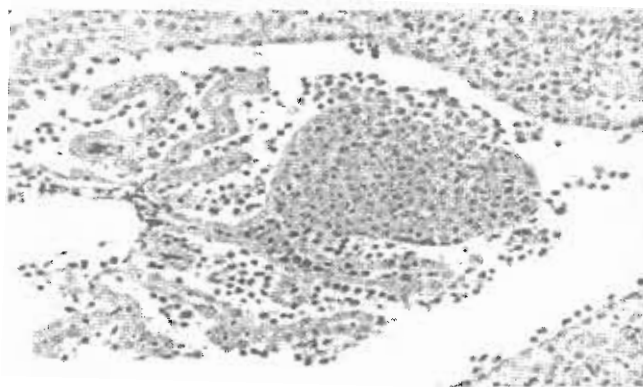


Fig. 6: Brain of mice from the control group, showing the neoplastic cells of D-GBM infiltrating the choroid plexus in the third ventricle (H&E X200).

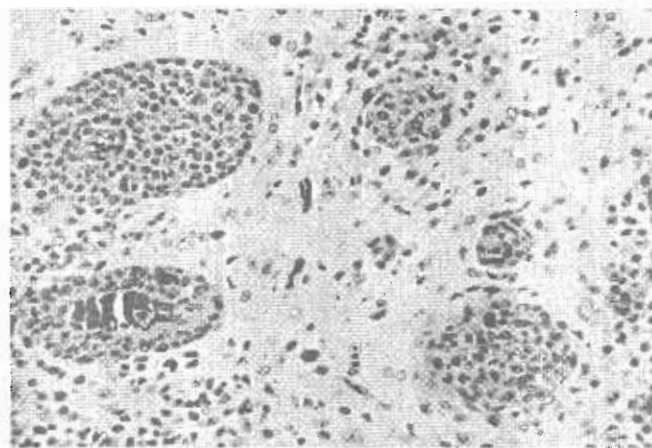


Fig. 7: Brain of mice from the curcumin group, showing the tumor cells surrounding the pre-existing capillaries (H&E X200).

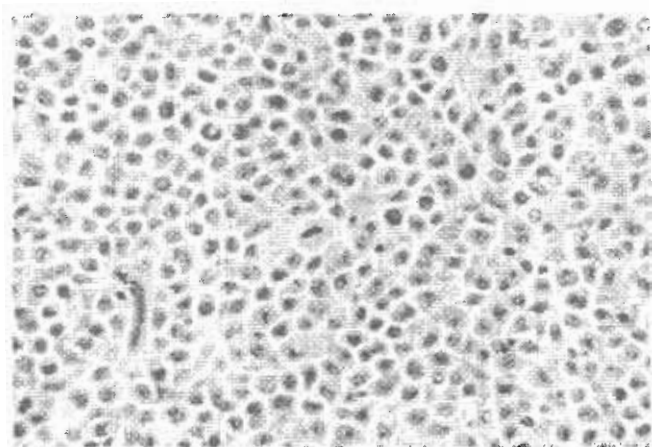


Fig. 8: Brain of mice from the control group, showing large polygonal tumor cells with distinct cell border also note the mitotic figure (arrow) (H&E X400).

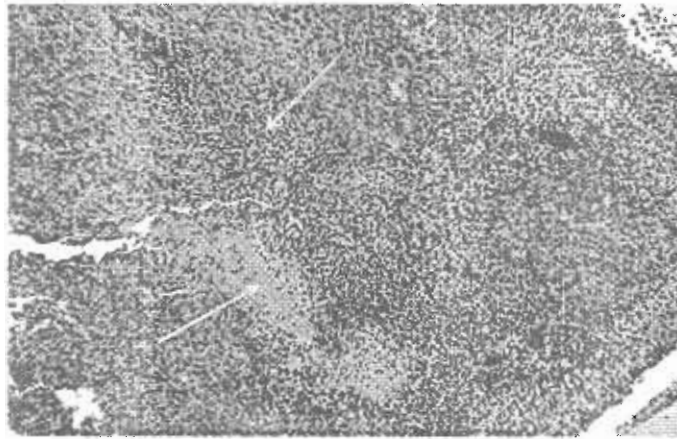


Fig. 9: Brain of mice from the control group, showing large area of necrosis within the tumor (arrows) (H&E X100).

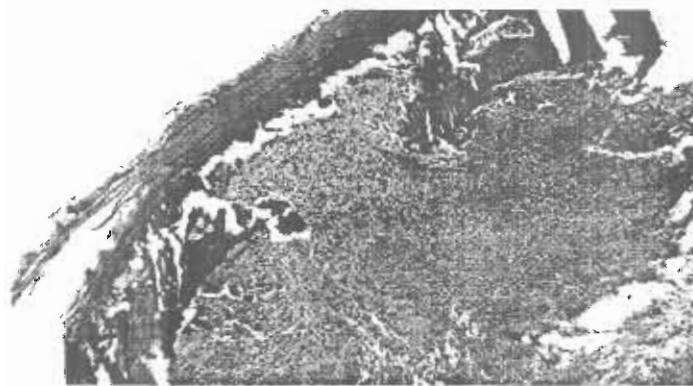


Fig. 10: Brain of mice from the Meriva group, sections showing the tumor infiltrating the skull bone (H&E X100).

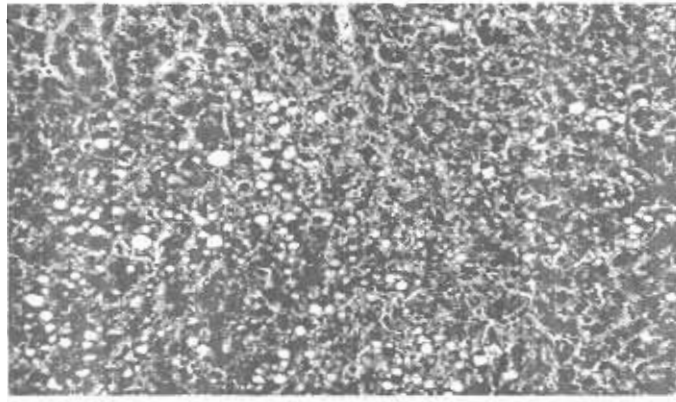


Fig. 11: liver of a rat administrated ccl4 without treatment, showing hepatic vacuolation and congestion of the sinusoids (H & E X200).

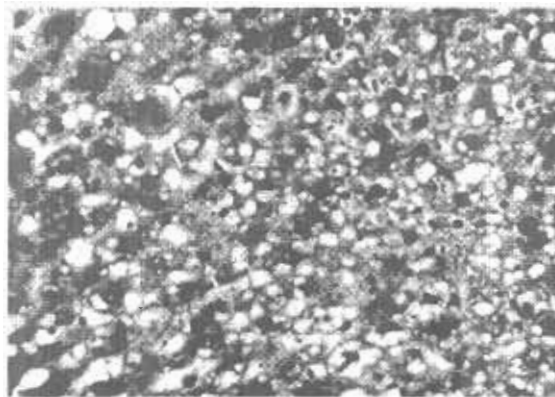


Fig. 12: Higher magnification of the previous image showing hepatic vacuoles displaced the nuclei to the periphery and ghost cells (arrows) indicative of necrosis (H & E X400).



Fig. 13: liver of a rat administrated ccl4 without treatment, showing congestion of portal vein (H&E X40).

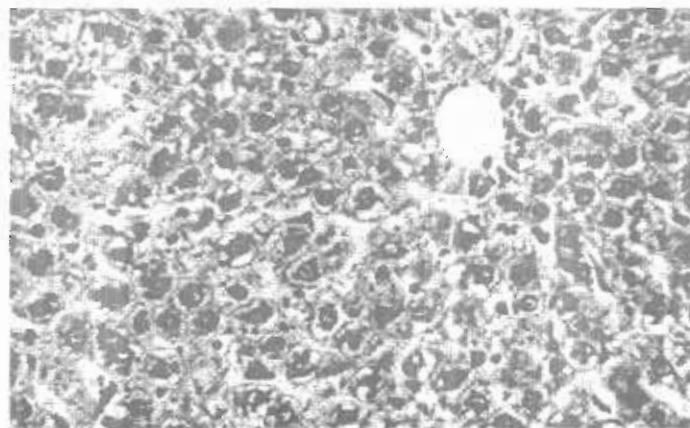


Fig. 14: liver of a rat administrated ccl4 and treated with camel's milk, showing mild hepatic vacuolation (H&E, X 400).

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