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Review Article

Natural Product Testing: Selecting in vivo Anticancer Assay Model

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ABSTRACT

Phytochemicals and other natural products have been suggested to be effective adjuvants to conventional therapy to reduce potential side effects that arise from cancer treatments. Various natural compounds and synthetic analogues have been studied for their potential anti-cancer properties in the last decade. Prior to administering them to patients in a clinical setting, drug development necessitates a sequence of preclinical testing, beginning with in vitro and progressing to *in vivo* studies. While numerous drugs and plant extracts were reported to be active *in vitro*, only several of them had reproducible activity at the tested doses *in vivo*. Furthermore, breakthroughs in manipulating gene editing have aided scientists in accurately replicating human diseases in animal models. Therefore, during drug development, *in vivo* studies are of tremendous help in evaluating a drug candidate's safety, toxicity, and efficacy in complex physiological and biochemical states. Hence, for this purpose, it is crucial to apply and select the animal model that is the most suitable to represent the studied disease or biological process. The current review summarizes various animal models that have been considered for *in vivo* testing, their advantages and disadvantages, and the experimental guidelines for conducting animal studies.

Keywords: Animal model, Anticancer model, In-vivo studies, Natural compounds

Introduction

The adaptation of plants and other microbes to predators and climate change through millions of years has diversified their secondary metabolites. Even so, only 10% of the world's natural resources have been explored for their potential medicinal uses [1]. Metabolites from plants, fungi, and bacteria are progressively tested for their anticancer properties [2] beginning with a large-scale screening programme in 1950 by the National Cancer Institute (NCI) that led to the discovery of paclitaxel from *Taxus brevifolia* and the synthesis of taxol [3]. In one report on drugs originating from natural products between 1981 and 2014, 174 new drug candidates for cancer treatment were approved by the United States Food and Drug Administration (U.S. FDA) and similar organisations.

Among them 22% were of natural product derivatives, 14% were of synthetic origin with the pharmacophore obtained from natural products, and 10% were unaltered natural products [4]. Drugs such as vincristine, irinotecan, etoposide, and actinomycin D have revolutionised medicine by bringing down the morbidity and mortality rates of cancer patients as with the consumption of phytochemicals which has also been proven to reduce the risk of contracting this disease [5]. In order to market compounds from natural products as a safe and effective anticancer treatment, precli-

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nical studies, which comprise several in vitro and in vivo experiments, need to be conducted prior to clinical trials and regulatory approvals. *In vitro* refers to tests which are carried out in a regulated environment outside of a living organism by using biological molecules, microorganisms, or cells in labware, while *in vivo* pertains to tests which are done within a whole living body, usually an animal model [6, 7].

Progress in method development for both *in vitro* and *in vivo* studies has enabled a better understanding of how natural products affect and halt the progression of cancer cells by reducing cell proliferation, arresting the cell cycle, inducing apoptosis, inactivating carcinogens, and acting as antioxidants [8]. The overall effect of these overlapping mechanisms helps slow the carcinogenesis process by aiming for multiple molecular targets and signal transduction pathways, such as the transmembrane receptor kinases and cytoplasmic kinases [9,10]. Therefore, the accuracy of preclinical data is vital to properly assess the efficacy, toxicity, pharmacokinetic, and safety of pharmacological leads from natural products before introducing them to the bedside [8]. The combination of both studies has also been utilised in in-vitro/in-vivo correlations (IVIVC), whereby the in vitro release test of a dosage form is related to its *in vivo* pharmacokinetic tests using mathematical models to predict the bioavailability and bioequivalence (BA/BE) outcome and monitor the drug stability [11].

In vitro tests are the critical first step in testing experimental hypotheses. Moreover, as they look into the low-key level of biological organisation, these tests provide useful information for tailoring *in vivo* experiments by breaking down complex biological systems of a living organism into many easily examined components [6]. In cancer research, the incorporation of primary cell cultures and continuous cell lines is crucial for the study of carcinogen identification, molecular mechanisms of tumour growth and metastasis, or drug screening and treatment development. Over the years, *in vitro* anticancer and chemoprevention models have undergone a major transformation from merely being a screening method to investigate cell proliferation, invasion, and cytotoxicity to being able to encapsulate the dynamic of the metastatic process through a better understanding of intravasation, extravasation, angiogenesis, matrix remodelling, drug delivery, and cell dormancy by using three-dimensional (3D) models of the tumour microenvironment [12]. As the output parameters get more expanded, *in vitro* studies are starting to imitate animal models in terms of the range of data presented, and this is, in a way, an advantage since the setup for in vivo experiments is more expensive and usually raises ethical concerns [6, 13].

Nevertheless, it should be noted that challenges such as different responses to treatment, side effects, and drug resistance among cancer patients due to genetic variability have always inconvenienced the oncologists and pushed them to come up with a more personalised treatment for individual patients [14]. Understanding pharmacogenetics and gene polymorphism allows for the tailoring of appropriate medications at optimal doses, and for this, animal models are the most suitable [15,16]. Furthermore, cancer is now being perceived as a systemic disease whereby the condition of its microenvironment is greatly influenced by blood vessel formation, immune cell function, and nutrient availability, which is hard to simulate using *in vitro* models even with the advanced technologies we currently have at hand for 3D cell culture [13,17]. In order to test the pharmacological properties of new drug leads from natural products, which include their efficacy as well as potential toxicity towards normal cells, an animal model may provide a comprehensive assessment.

Aside from this, *in vitro* data have been shown to have some discrepancies with the corresponding *in vivo* results of the same study, owing to the efficacy of the tested products being reduced by the metabolic processes in animal models [18]. Dehydrogenation, hydrogenation, hydroxylation, demethylation, glucuronidation, dehydration, and glycosylation are some of the common pathways in which lead compounds from natural products may undergo changes to their structure before they remain in the plasma or be excreted through the urine, bile or faeces [19]. Studies that probe the reduced efficacy of natural products within an organism, for example, from one review which proved that the bioavailability of resveratrol is lower *in vivo* than *in vitro* due to the rapid metabolism of this polyphenolic compound to its conjugate forms [20], are beneficial not just to prevent the extrapolation of *in vitro* data to *in vivo* conditions while ignoring other confounding variables but also to analyse the individual efficacy of these

conjugates as some are more bioavailable and efficacious than the parent compound [21, 22]. The bioavailability of natural products eventually determines the pharmacokinetics and thus the safety and efficacy levels of the lead compounds; therefore, it is crucial to fully understand the limiting and promoting factors pertaining to this using an animal model with metabolic processes as close as the humans as to reach a thorough evaluation. There has been ample evidence of the widespread preference for the animal model in studying the effects of natural products on cancer outcomes. A few of such studies are of the mushroom *Inonotus obliquus* anti-inflammatory and antiproliferative properties against adenoma and colitis-associated colon cancer mouse models [23], *Scutellaria barbata* potential in suppressing angiogenesis in Athymic BALB/c nu/nu male mice by targeting the Sonic Hedgehog (SHH) pathway [24], boswellic acid, a compound derived from *Boswellia serrata,* anticancer activity against colon cancer as tested in nude mice with orthotopically implanted tumour [25], and even in cancer prevention study using cactus pear aqueous extract in nude mice which later successfully increased annexin IV expression and decreased vascular endothelial growth factor (VEGF) expression in them [26]. Animal models are also useful in exploring the immunostimulatory effects of natural products to support their use as an immunotherapeutic agent in cancer treatment [27].

Regardless of the different attention in cancer research, the final goal is always to develop a cure for cancer. Using the advanced knowledge and technology today in developing a specified *in vivo* model in relation to the study objectives, it is hoped that more lead drugs with potent anticancer properties from natural products can be discovered, and the survival of cancer patients can be greatly improved as a result of that. Therefore, the correct selection of *in vivo* models that closely mimic the pathophysiology of different types of cancer in humans is desirable for an accurate research outcome.

Animal Cancer Models

The choice of the right animal model to be used in *in vivo* experiments depends on multiple factors. Various models have already been developed and used over the centuries for cancer research, and these can be broadly classified into homologous, isomorphic, and predictive. Homologous indicates a model that showcases every aspect of a medical condition from cause to symptoms and treatment, isomorphic is similar to homologous except that the cause is not inflicted in a similar way as in the humans, and the predictive model is used when the effect of certain treatments needs to be investigated [28].

These models are essential in understanding the complex biochemical and physiological processes involved in cancer initiation and progression, as well as the effect of treatment in an organism. In drug discovery research, investigating the pharmacodynamic, pharmacokinetic, and other pharmacological information of the lead compound will aid in the identification of a novel therapeutic agent, its possible combination or adjunct property with other existing chemotherapy drugs, and the mechanism of cytotoxic or targeted therapies resistance from the biophysical, molecular, cellular, and behavioural standpoints [28, 29]. There is no single model which fits all experiments, and therefore, the choice for the most suitable one greatly depends on several aspects like ethical concerns, availability of the mouse strain, tumour compatibility, the purpose and severity of the procedures performed on the animals, restrictions such as genetic variability and immune status of the animals used, financial and logistic resources, and the time available for the completion of the project [30, 31].

As for the species of animal for *in vivo* study in cancer, mice, rats, zebrafish, pigs, monkeys, dogs, and rabbits are the usual candidates that have been utilised thus far [32]. However, among all, the murine animal model was favoured in 95% of cancer studies [33] for it is widely available at a low cost and caters to diverse immunocompetent and immunodeficient strains, but above all, mice and humans share many genomic and physiological characteristics of tumour biology which makes this model a desirable choice for its application in translational medicine [34]. The first mouse cancer model, which is also known as the oncomice, was genetically engineered to express its dominant oncogenes back in the 1980s. This was achieved by combining two growing areas of research at that time, which were gene cloning in animals and molecular cloning of viral and cellular oncogenes [35]. Eventually, the invention of transgenic mice paved the way for more modifications in the animal genome with the emergence of knockout and knock-in mice along with new efforts in producing

a murine cancer model with a higher degree of tumour heterogeneity to match that of the humans' [34].

In natural product research, the use of murine model for *in vivo* study is widespread for its importance, and the discovery of many promising anticancer extracts and compounds from these studies has further driven researchers to search for more new drugs and closely study their mechanism of anticancer actions [36]. The success in combating cancer cells for naturally derived compounds such as dihydroartemisinin, artesunate, coptisine, isoegomaketone, and oridonin, was proven and carefully outlined using a range of mice models, namely tumour-bearing and tumour-nude mice and other strains such as C57BL/6, BaLB/c, and MTLn3, among others [37]. *In vivo* experiments using the murine model are capable of answering many questions pertaining to tumour growth, survival time, the degree of remission or cure, and the drug's mechanism of action, efficacy and safety [31].

Causal studies, for one, help in determining the predisposing and triggering factors for the development of cancer cells in mice and how prophylactic intake of natural products may hamper this unwanted outcome by acting as an anti-inflammatory, antioxidant, anti-hormone, and immune-enhancing agent [38][39]. One study proved that inflammation in obese C57BL/6 mice may increase the risk of developing colorectal carcinoma [40], and another claimed that vitamin D deficiency permits breast cancer cells to grow in the bones of non-tumour bearing nude mice, as driven by the secondary changes in the bone microenvironment [41].

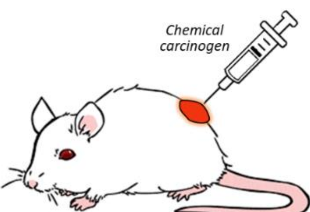
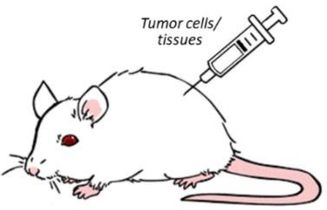
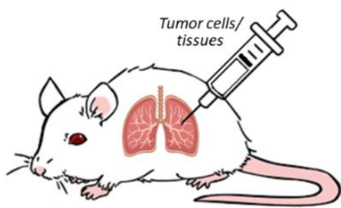
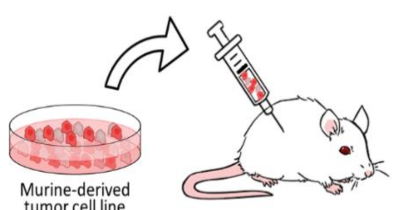
Aside from this, another appeal of *in vivo* studies is in the discovery of biomarkers which are used to determine cancer diagnosis, prognosis, progression, remission, recurrence, and response to therapy [42]. NCI of the United States government states that a biomarker is a biological molecule found in blood, other bodily fluids, or tissues that is a sign of a normal or abnormal process or a condition or disease [43]. As protein detection gets more advanced with the help of spectrometric methods, biomarkers such as cathepsins B and D (colon cancer), fibulin and osteopontin (breast cancer), and Insulin-like growth factor-binding protein (IGFBP2), Tissue inhibitor of metalloproteinases 1 (TIMP-1), Retinoic acid receptor responder protein 2 (RARRES2), Cluster of differentiation 14 (CD14) and Granuli (GRN) (ovarian cancer) were able to be discovered and applied in the diagnosis and monitoring of cancer progress [42]. Although discovering novel cancer biomarkers is more difficult in humans than in mice due to the genetic and environmental heterogeneity in the former, mice with their homogenous tumour will provide valuable insights into understanding the nature of various cancers, and this can be modulated accordingly for the studies on humans at a later stage. The usefulness of biomarkers study is undisputable in the detection of early cancer lesions, which can be incorporated in the cancer screening of the mass public or at-risk patients as it requires minimal intervention or invasion [44]. A few examples include the detection of microRNA-196a and -196b for the early detection of familial pancreatic cancer using KPC mice [45], and another is on the early diagnosis of cervical cancer by detecting the minichromosome maintenance protein 7 (MCM7) using K14E6 and K14E7 mouse strains [46].

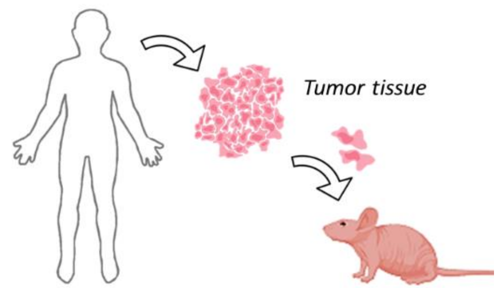
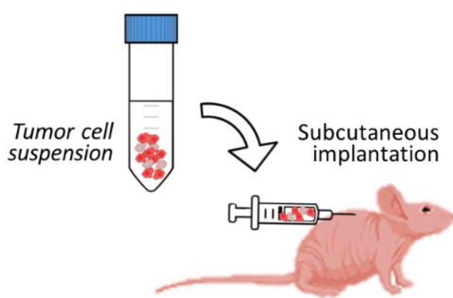
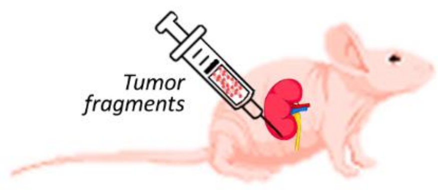
In addition to that, the other method for early cancer detection lies in the imaging technique, for which the murine model may help advance its technology and accuracy. Many approaches have been employed to aid in the visualisation of mouse tumours, and these include micro–positron emission tomography, single-photon emission computed tomography, microcomputed tomography, bioluminescence imaging (BLI), magnetic resonance imaging, whole-body fluorescence imaging, intravital microscopy, and ultrasound [47]. The progress of transgenic technology has also given rise to reporter mice with fluorescent, bioluminescent proteins or biochemical tags being inserted into their genome for real-time monitoring of the cancer cells and molecules [48].

Natural product research mainly concerns the efficacy and safety of the pharmaceutic preparations before introducing them in clinical trials. For this, employing a clinically relevant and molecularly characterised murine model is essential [42]. Many studies have proven the usefulness of *in vivo* study using mice to understand better cancer response to naturally-derived compounds such as apigenin, recursion, gingerol, nimbolide, and pterostilbene, and some them like lycopene, quercetin, berberine, and curcumin have already been introduced in clinical trials due to their promising success at preclinical stage [8]. Studies on the mechanism of action of these anticancer agents are also

Table 1. Summary of animal models of cancer

Mouse model	Characteristics	Types of cancer	Method of implantation	Ref.
Spontaneous tumour model	Animals that naturally develop cancer like mice of some inbred strains	Leukemia, mammary gland tumour, pulmonary adenomas, hepatoma		[50]
Virus induced tumour model	Transfection of pre-implanted embryos with retroviruses which contain genes that will be replaced or modified	Leukemia (e.g.: Friend leukemia)	-The virus is transmitted by injecting cell-free filtrate from leukemia-spleen homogenate	[50]
		Skin tumour (e.g.: Rous sarcoma)	-The virus spreads by implanting tumour fragments or inoculating cell-free material from tumour homogenate	
Radiation induced tumour model	Used to cause cancer by exposing the laboratory animals to identified radiation dosages	Skin tumour	-By exposing the experimental animals to ultra-violet (UV) ray using predetermined doses of radiation -Sometimes radiation is utilized together with a chemical agent like TPA or DMBA	[50]
Chemically induced tumour models	Tumours caused by chemical carcinogens derive from the host cells	Mammary gland, colon tumour	-Chemical carcinogens can be classified into two categories: i. **Direct acting agents**- require no chemical transformation to cause carcinogenicity ii. **Indirect acting agents**- become active only after metabolic conversion. Also called, procarcinogens	[50][51]

			and their active end products are called ultimate carcinogens  	
Transplantable tumour model	Depends on the use of cancer cell lines or tissues that may grow in mice or rats		-Two methods of transplantation: **i. Heterotopic transplantation**- transplantation of tumour cells or tissue at a site other than its place of origin using intraperitoneal (i.p.) or subcutaneous (s.c.) routes  **ii. Orthotopic transplantation**- transplantation of cancer cells to the anatomical location or tissue where the tumour is produced (e.g.; lung tumour is transplanted in the lungs)  	[50][33]
	Divided into two broad groups which are the cancer origin and the host	Lewis lung carcinoma, Ehrlich ascites carcinoma	-Two types of models: **i. Syngeneic model**- the use of mouse or rat cancer cell lines or tissues for transplantation into inbred animals with the same genetic background as the derived cell lines or tissues by i.p. injection of 10^5 leukemic cells 10 or s.c. transplantation of the solid tumour fragments into the flank area  	

	CX-1 colon, LX-1 lung, MX-1 mammary cell lines	**ii. Xenogenic (xenograft) model-** tumour cell lines can be transplanted into an athymic (nude) or severe combined immunodeficient (SCID) mice via multiple routes like s.c., i.p., intravenous, intracranial, intrasplenic, renal subcapsular, or through a new orthotopic model by site-specific organ inoculation 
Subcutaneous implantation		-A tumour cell suspension (approximately 10^6 to 10^7 cells per animal) is usually injected into the flank region of the mouse -It normally takes a few days to a few months for tumours to develop, depending on the growth rate of the cell line used 
Renal subcapsular (RSC) assay		-The cells in the form of tumour fragments are inoculated into a nude mouse (1 mm in size and located under the capsule of the kidney) 
Intraperitoneal, microencapsulated tumour assay		-Tumour cells are encapsulated in semi-permeable gels that can be in a microcapsule form (0.05 to 1 mm).

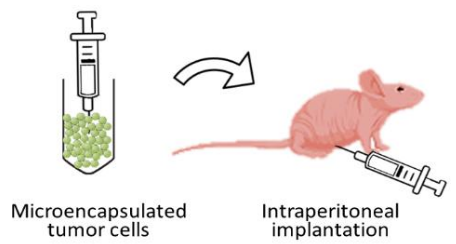
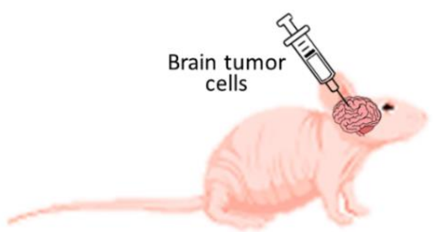
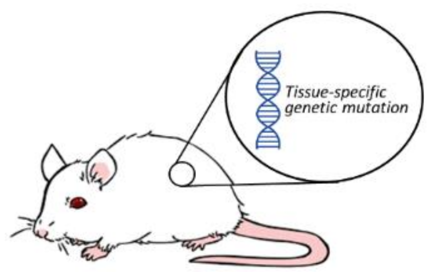
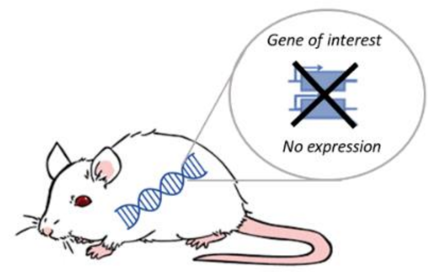
			-They can be inoculated in the peritoneal space 	
	Orthotopic xenograft model		-Implantation of tumour cells at the site of the originating organ 	
Genetically engineered mice (GEMs)	Transgenic mice	Breast, prostate gland, mammary gland, colon tumours	-Resulting in progeny of the pronucleus of a fertilized egg that is injected with a foreign gene -This progeny then carries and expresses this exogenous gene and passes it on to its offspring -Possible transfer of genes to the pronucleus by microinjection, retroviral infection, or embryonal stem cell (ESC) transfer 	[50][51]
	Knockout mice	Prostate gland, mammary gland tumours, lymphomas	-An animal model created by omitting both alleles of a specific gene. 	

Table 2. Advantages and disadvantages of different animal models of cancer

Mouse model	Subtype	Advantages	Disadvantages	References
Spontaneous tumour model		-Closely mimic the clinical condition -Corresponds to human cancers in its kinetics and antigenicity -Plays key roles in cancer and carcinogenic molecule research	-Inadequate amount of tumour cells for assessment purposes -Not replicable, and most of them have viral origin	[50]
Virus-induced tumour model		-Inhibits spleen weight gain, reduces live virus titre, and prolongs survival time -The assessment of the tumour growth is straightforward	-Laborious and lengthy evaluation of parameters -Not sensitive to many types of agents; hence, a few numbers of essential compounds may escape	[50]
Radiation-induced tumour model		-Tumours grow on the skin, making it easy to assess them -Can be used to predict a general antitumor activity depending on the evaluation parameters	-The utilisation of radiation may cause a radiation risk to researchers -Long tumour induction time and time-consuming evaluation criteria for assessment -Not used in practice for screening procedures	[50]
Chemically induced tumour models		-Resemble human clinical cancer more closely than transplantable neoplasms	-Chemically induced tumours are the possible effects of carcinogen upon the tumour behaviours -Hazards to other species and workers towards the excreted carcinogen and its metabolites that may present in the faeces and urine of the animals	[50, 51]
Transplantable tumour model	Heterotopic transplantation	-The inoculation procedure is simple and less time-consuming -It is possible to inoculate many animals at a time -Involves less skill		[33, 50]
	Orthotopic transplantation	-Strongly resembles human cancers, including tumour histology, vascularity, gene expression, chemotherapy response, and metastatic biology -Orthotopic cancer cell transplantation can be performed by direct injection of tumour cells or by surgical in situ implantation (SOI)		
	Syngeneic model	-Close interaction between tumour and host	-Fast-growing leukaemia cells may be beneficial in	

		-1st stage pre-screening in the NCI Drug Screening Program from 1955-1985 -Low cost and allows for a higher compound production -It is a widely used model for primary screening procedures as it can predict general anti-tumour activity	selecting compounds that are active against fast-growing tumours -Lower rates of metastasis
	Xenogenic model	-Maintain original morphologic and biochemical characteristics -Mainstay of cancer drug discovery programmes -Human-origin transplantable tumours should be used as they resemble the clinical diseases more closely	-Severe immune rejection may result from transplantation of such human tumours in mice
	Subcutaneous implantation	-Simple and gives easy access to tumour -NCI has included this method as the primary *in vivo* drug discovery and screening test	-Invasion in adjacent tissues and metastasis is rare with subcutaneous xenografts
	Renal subscapular (RSC) assay	-Maintains true morphologic, functional, and growth features of the original tumour, such as tumour cell contact and spatial relationship -Better characterises the metastatic properties of human tumours -The time between tumour inoculation and the appearance of a palpable mass is relatively short and constant -The tumour can usually be evaluated within six days; therefore, this model is particularly suitable when short-term *in vivo* measurements are required -Ideal orthotopic model for renal cell carcinoma	-Not the optimal model since a fully immuno-privileged site is not the subcapsular region of the kidney -Invasion of variable amounts of lymphocytes demonstrated by tumours in this region, possibly due to a contradictory factor from the original tumour
	Intraperitoneal microencapsulated tumour assay	-The microencapsulation assay is simple, rapid, and relatively inexpensive -Compared with subcutaneously transplanted	

		tumour detection, fewer mice are needed -Adaptable to most solid tumours and use immuno-competent mice -More than one tumour in the same mouse can be measured simultaneously -Being tested as an *in vivo* second-line screen by the NCI screening program		
	Orthotopic xeno-graft model	-Surgical orthotopic implantation (SOI) models have greater relevance to clinical metastatic cancer -It provides the best growth and progression environment for tumour cells -To be used widely to explore its function as a model of *in vivo* assessment of cytotoxic agents	-It has not been used widely by the NCI drug-screening program.	
Genetically engineered mice (GEMs)		-Outstanding models for studying the oncogenic phenotype that results from the dysregulation of a known gene -Resembles human cancer better than the other models outlined because the tumour develops spontaneously in its natural organ -The tumours have a natural growth rate and metastatic characteristics which is identical to their natural history in humans	-These tumours are non-immunogenic in the natural host; therefore, they overcome the need for immunosuppressive animal growth	[50, 51]

crucial to accurately deliver the drug to its appropriate site or molecular targets. Interference of the MAPK signalling pathway, suppression of ATK signalling, and disruption of cell cycle progression are some of the effective ways the progress of cancer cells can be halted [49]. Therefore, it is crucial to understand the pathophysiology and natural history of different types of cancer in order to choose the mouse model which best suits the objective of the interventional study. Table 1 summarises the different types of mouse models, their characteristics, and the method of cancer cell implantation to provide a simplified guideline for choosing the best in vivo experimental design, while Table 2 outlines their respective advantages and disadvantages.

Experimental Design

The proper treatment and use of laboratory animals in research, testing, teaching and production demand scientific and professional assessments on the basis of the animals' needs and applications. The Institutional Animal Care and Use Committee (IACUC), an animal experimental protocol ethical review body, has various guidelines pertaining to this matter. Each institution should set up a committee to ensure that animal care, facilities and use for scientific purposes are well taken care of [33]. To represent scientific and non-scientific interests, the scope and design of this committee are sufficient. The monitoring of animal care and use is performed by a committee review, regular program reviews, and facilities inspection of

1. Animal type and number
2. Justification for the use of animals and proof that organisms and statistics are appropriate
3. Comprehensive project overview
4. Whether suggestions prevent or reduce stress, pain or discomfort
5. Where 3Rs are complied with, i.e. substitution through non-living structures, decrease of numbers or refinement of methods
6. That past experiments are not unnecessary duplicated
7. These animals are not repeated endlessly unless justified
8. Adequate description of human endpoints and justification
9. Appropriate drug use (sedatives, analgesics or anesthetic) and techniques in consultation with the attending veterinarian
10. Unless anesthesia is coupled, paralysis will not be used.
11. Qualifying and training animal procedures staff.
12. Adequate and proper care of husbandry for animals
13. Appropriate protocols, techniques and care of surgery before, during and post-surgery
14. Euthanasia Methods

Figure 1. Important areas identified by IACUC in reviewing proposals involving animal

proposed activities using vertebrate animals. The committee reviews then approves or rejects proposals concerning animal use, considering ethical, social, scientific, and educational values [52]. IACUC has identified several essential areas when reviewing animal use proposals, as shown in Figure 1.

Successful experimental design is of fundamental importance since inadequately planned experiments produce poorly supported results. Critical components of experimental design are the representation of controls, treatment plans, group sizes, and randomisation protocols. If these factors are not considered, it is possible to overestimate or underestimate the effectiveness of the new chemotherapeutic agent. The potential influence of the studied formulations on the experimental findings should also be tackled because the formulation itself may directly impact the tumour or the host, such as unexpected toxic side effects [53]. Furthermore, experimental manipulation of animals may cause stress reactions, significantly affecting

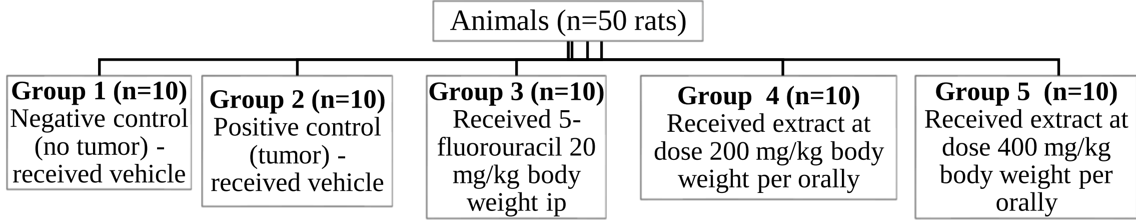


Figure 2. Experimental design and classification of animal group. There are five groups containing 5-10 mice (n=5-10) with a similar weight in each group. Each animal is implanted at designated site with approximately 1 x 10^6 cells cancer cells of choice into a tumour community. Treatment begins when the tumour reaches a predetermined size.

the findings.

Animals treated with vehicles should also be used as experimental controls rather than untreated animals. However, this raises the overall number of experimental animals that need to be cared for during the study [54]. An example of an animal study design for tumour induction and validation of drug exposure can be set up as illustrated in Figure 2 [55].

This serves as a guide and varies depending on the study's objectives. Pharmaceutical companies have recognised and utilised many endpoints for subcutaneous tumour models to improve their drug development operation. Under the US National Cancer Institute's Developmental Therapeu tics Program (http://dtp.nci.nih.gov; accessed on 31st August 2022), subcutaneous tumour endpoints include percentage test/control (percentage T/C), tumour weights calculated on each day, tumour growth delay, net log cell kill, median days to given tumour weight or stated number of tumour doublings, and tumour regression. The goal of the research is to determine the effects of development phase exposures on tumour volumes [53].

These endpoints are based on the measurement of the length and width of the tumour. Calliper measurements are subjective, and in this case, skin thickness would significantly affect the results; therefore, any measurement that is less than 63mg of the tumour demands conviction, and whenever possible, ultrasound should be used instead [56]. Emphasis on precise and reproducible measurements must be included in the experimental protocol so that the information obtained can be correctly processed and analysed [57]. For example, the same criteria must be used to consider all tumours irrespective of whether they have been treated with a vehicle or test agent. The scale can also affect the accuracy of the weight. To remove resection bias, tumours growing within the organ can be evaluated by resetting and weighing the entire organ. This method can only be used if the time between tumour inoculation and measurement has elapsed in such a way that a large mass. The tumour exists to provide statistically significant differences between the treatment groups [53].

Alternatively, visceral lesions may be assessed by histopathological assessment either via manual or automated quantification of the number or volume of lesions. Quantifying the number and size of the lesions may provide an unbiased endpoint in cases where multiple metastatic lesions occur. In addition, the number of microscopic lesions is a common endpoint for metastase models. With the advent of modern imaging techniques such as bioluminescence, ultrasound, and magnetic resonance imaging, more improved and highly sensitive methods are used to evaluate the growth of visceral tumours to complement other traditional tumour assessments [58].

In summary, it is important to choose a reproducible and accurate endpoint because the data produced from the *in vivo* studies is used to make critical decisions on the development of new drugs [53].

Downstream Analysis

The ultimate goals of any anticancer treatment are to minimise the tumour burden, reduce tumour-related morbidity, increase the quality of life of the patients, and, when possible, improve their life span. Therefore, the anticancer efficacy of newly discovered drugs can be defined in various ways [59]. The effectiveness of treatment must be monitored by a systematic process so that the treatment outcome can be measured repeatedly.

When testing for *in vivo* efficacy, a number of factors must be considered: the route of administration of the test substance, the dose used,

experimental reading (specific and sensitive) and the use of positive controls, if any. Factors such as physicochemical properties, pharmacokinetics and toxicological evaluation are equally important when selecting the drugs [60]. The target of the new generation of anticancer drugs is expressed in both human disease models and animal models. Observation of the following parameters could be used to assess the effects of the anticancer agent.

Acute toxicity

Toxic effects, behavioural alteration, locomotion, convulsion and mortality of the tested animals will be observed for 14 days.

Percentage weight increase as compared to day-0 weight

All animals will be weighed on the day of inoculation and once every three days during the post-inoculation period. The percentage increase in weight will be calculated using Equation 1.

$$Percentage\ increase\ in\ weight\ (\%) = \left[\left(\frac{animal\ weight\ on\ a\ given\ day}{animal\ weight\ on\ day-0}\right) - 1\right] x\ 100\%$$

Tumour size

Excision and weighing tumours at the end of the experiment can give an extra endpoint while avoiding mistakes caused by tumour shape fluctuations and volume or mass predictions prior to this. The use of callipers (two diameters at right angles to each other) to measure the size of superficial tumours is a simple and precise procedure.

Median survival time (MST) and percentage increase in lifespan (ILS)

All animals will be carefully monitored, and the time of death of each mouse will be recorded. Percentage increase in life span is calculated as in Equation 2.

$$Percentage\ increase\ in\ lifespan\ (\%) = \left[\left(\frac{MST\ of\ treated\ group}{MST\ of\ control\ group}\right) - 1\right] x\ 100\%$$

The median survival time (MST) is calculated by adding the time of the first death and the last death and then dividing the value by two.

Haematological parameters

The effect of the pharmaceutical preparations on the haematological parameters of tumour-bearing mice will be evaluated on the 14th-day post-transplantation between the treatment and control groups. Each mouse will be anesthetised, and blood will be drawn out from its retro-orbital plexus. The total number of white blood cells (WBC), red blood cells (RBC), and the haemoglobin level will be analysed.

Histopathological evaluation

Liver and spleen tissues will be harvested for histopathology assessment to determine any toxicological effect of the tested drugs or preparations. The impact of pre- and post-mortem sampling must be considered as part of the decision on sampling methods, as some molecular targets may remain stable within a few minutes after death, while others may degenerate rapidly when respiration and perfusion cease. If the endpoint of the assessment cannot be effectively measured in this condition, sample collection can be done under general anaesthesia [53]. Biopsy needles, which are commercially available, can be used to collect samples, and they can be preserved in situ by freezing. If in situ freezing is not possible, samples obtained by needle biopsy or resection can be placed in a liquid fixative (for example, 10% neutral formalin and RNA later, Ambion, Austin, Texas) and can then be quickly frozen by transferring them to a pre-frozen cryotube or stored in other practical ways [61]. Regardless of which method is selected, the preservation process must be consistent for all samples. It is suggested that factors such as fatigue faced by the operator when processing huge samples should not be neglected so that the relative collection time does not affect the study outcome.

If the study goal is to determine how molecular markers are regulated, then the experimental design must establish which samples should be collected for analysis. Useful samples can be tumour tissue, replacement tissue (for example, spleen, bone marrow, and skin), serum, or plasma. Regardless of the experiment carried out, a key factor in its design is the sample selection time after being exposed to the test agent. As several markers are unstable and may change due to experimental conditions, it is also important to collect and store the samples accordingly to obtain reliable results [53][62]. Methods of collection such as frozen biopsy, conventional needle biopsy, and complete or partial tumour resection should be scientifically determined through analytical and optimization studies until the final treatment plan

is completed [53].

Furthermore, it is also necessary to consider the appropriate sample size, the number of tissues required to conduct the study, and the effect of the endpoint stability on the sample selection technique. Efficacy data can be used to assess the differences between the treated and control groups and evaluate the significance of the independent variables as well as the specific conditions of the experimental procedure [63]. The statistical tests used will depend on the experiment and its objectives.

There are several different ways to choose a method of statistical analysis, but during the development of the experiment, the most appropriate approach is to obtain the assistance of a trained statistician. Regardless of the type of data, selecting the data analysis statistical evaluation and defining the criteria for removing outliers before the experiment is conducted will ensure a quality data analysis and data interpretation [59]. An apparent example of this form of bias occurs when tumours which do not grow in the control group are omitted from the study, while tumour-free animals are included in the treated groups because the researcher expected the treatment to work [53].

Conclusion

The use of animal models has paved the way in the field of drug discovery from natural products, especially in helping us better approach the efficacy and safety issues of new drugs before introducing them to humans in clinical trials. Several important criteria, like the selection of relevant experimental design, a suitable animal model that includes the species, strain, and tumour type, as well as the execution and evaluation of the *in vivo* experiments, have to be established. In addition to some considerations of the potential overprediction or underprediction of the present models, efficiency studies and the decision to proceed with clinical trials should be done with caution. Ultimately, an improved animal model for cancer screening could reduce the cost and increase the pace of finding newer drugs to help us with the continuous battle of preventing and treating various types of cancer. It is hoped that more advanced technologies and innovative *in vivo* studies can help to bridge the gap between *in vitro* and clinical studies in the future.

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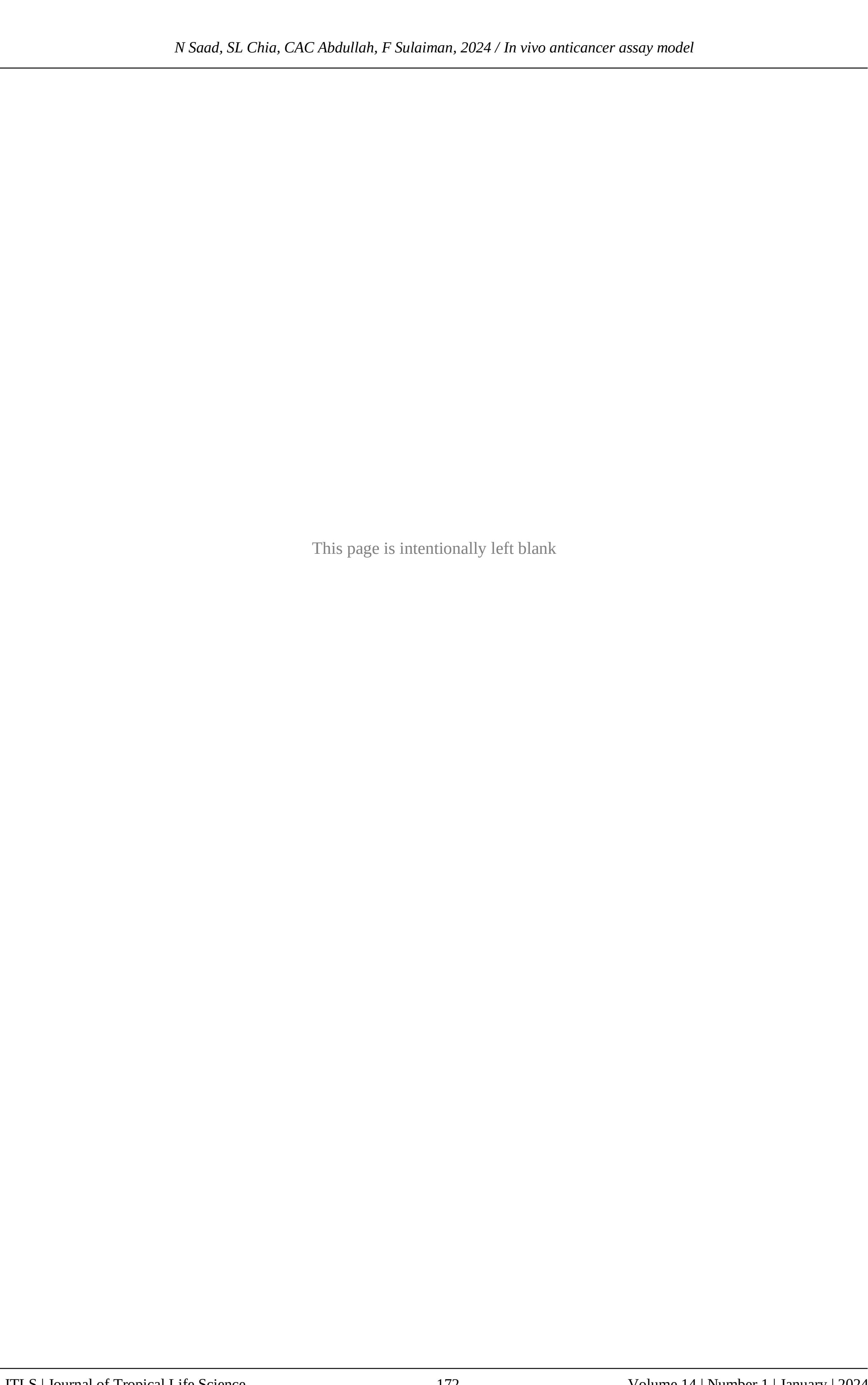
References

1. Amaral RG, Santos SA dos, Andrade LN, Severino P, Carvalho AA (2019) Natural Products as Treatment against Cancer: A Historical and Current Vision. Clinics in Oncology 4(1562):1–5.
2. Kinghorn AD, Chin YW, Swanson SM (2009) Discovery of natural product anticancer agents from biodiverse organisms. Current Opinion in Drug Discovery & Development 12(2):189–96.
3. YANG YH, MAO JW, TAN XL (2020) Research progress on the source, production, and anti-cancer mechanisms of paclitaxel. Chin J Nat Med. 18(12): 890-897. doi: 10.1016/S1875-5364(20)60032-2.
4. Newman DJ, Cragg GM (2016) Natural Products as Sources of New Drugs from 1981 to 2014. Journal of Natural Products79(3):629-661. doi:10.1021/acs.jnatprod.5b01055.
5. Ranjan A, Ramachandran S, Gupta N, Kaushik I, Wright S, Srivastava S, et al (2019). Role of phytochemicals in cancer prevention. International Journal of Molecular Sciences 2;20(20). doi: 10.3390/ijms20204981.
6. Quinn B (2014) Preparation and Maintenance of Live Tissues and Primary Cultures for Toxicity Studies [Internet]. Biochemical Ecotoxicology: Principles and Methods. Elsevier Inc; 33–47. doi :10.1016/B978-0-12-411604-7.000039.
7. McGonigle P, Ruggeri B (2014) Animal models of human disease: Challenges in enabling translation. Biochemical Pharmacology 87(1):162–71. doi: 10.1016/j.bcp.2013.08.006.
8. Choudhari AS, Mandave PC, Deshpande M, Ranjekar P, Prakash O (2020) Phytochemicals in cancer treatment: From preclinical studies to clinical practice. Frontiers in Pharmacology 10(January):1–17. doi: 10.3389/fphar.2019.01614.
9. Bianco R, Melisi D, Ciardiello F, Tortora G (2006) Key cancer cell signal transduction pathways as therapeutic targets. European Journal of Cancer 42(3):290–4. doi: 10.1016/j.ejca.2005.07.034.
10. Shu L, Cheung KL, Khor TO, Chen C, Kong AN (2010) Phytochemicals: Cancer chemoprevention and suppression of tumor onset and metastasis. Cancer Metastasis Rev. 2010;29(3):483–502. doi: 10.1007/s10555-010-9239-y.
11. Cardot JM, Beyssac E, Alric M (2007) In vitro–in vivo correlation: Importance of dissolution in IVIVC. Dissolution Technologies 14(1):15–9. doi:10.14227/DT140107P15.
12. Katt ME, Placone AL, Wong AD, Xu ZS, Searson PC (2016) In vitro tumor models: Advantages, disadvantages, variables, and selecting the right platform. Frontiers Bioengineering and Biotechnology 4(12). doi: 10.3389/fbioe.2016.00012.

13. Ravi M, Paramesh V, Kaviya SR, Anuradha E, Paul Solomon FD (2015) 3D cell culture systems: Advantages and applications. Journal of Cellular Physiology 230(1):16–26. doi: 10.1002/jcp.24683.
14. Coate L, Cuffe S, Horgan A, Hung RJ, Christiani D, Liu G (2010) Germline genetic variation, cancer outcome, and pharmacogenetics. J Clin Oncol. 2010;28(26):4029–37. doi: 10.1200/JCO.2009.27.2336.
15. Cirulli ET, Goldstein DB. In vitro assays fail to predict in vivo effects of regulatory polymorphisms. Human Molecular Genetics 15;16(16):1931-9 doi: 10.1093/hmg/ddm140.
16. Zhu F, Dollé MET, Berton TR, Kuiper R V., Capps C, Espejo A, et al. (2010) Mouse models for the p53 R72P polymorphism mimic human phenotypes. Molecular and Cellular Pathobiology 70(14):5851–9. doi: 10.1158/0008-5472.CAN-09-4646
17. Kareva I, Wilkie KP, Hahnfeldt P (2014) The Power of the Tumor Microenvironment: A Systemic Approach for a Systemic Disease. Mathematical Oncology 2013: 181–196 2014;181–96. doi: 10.1007/978-1-4939-0458-7__1.
18. Chen YY, Liang JJ, Wang DL, Chen JB, Cao JP, Wang Y, et al (2022) Nobiletin as a chemopreventive natural product against cancer, a comprehensive review. Critical Reviews in Food Science and Nutrition 63(23):6309-6329. doi: 10.1080/10408398.2022.2030297.
19. Sun E, Xu F, Qian Q, Cui L, Tan X, Jia X (2014) Ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry analysis of icariside II metabolites in rats. Natural Product Research 28(19):1525–9. doi: 10.1080/14786419.2014.921684.
20. Wenzel E, Somoza V (2005) Metabolism and bioavailability of trans-resveratrol. Molecular Nutrition and Food Research 49(5):472–81. doi: 10.1002/mnfr.200500010.
21. Liu Z, Hu M (2007) Natural polyphenol disposition via coupled metabolic pathways. Expert Opinion on Drug Metabolism and Toxicology 3(3):389–406. doi:10.1517/17425255.3.3.389.
22. Walle T (2011) Bioavailability of resveratrol. Annals of the New York Academy of Sciences 1215(1):9–15. doi:10.1111/j.1749-6632.2010.05842.x.
23. Mishra SK, Kang JH, Song KH, Park MS, Kim DK, Park YJ, et al. (2013) Inonotus obliquus suppresses proliferation of colorectal cancer cells and tumor growth in mice models by downregulation of β-catenin/NF-κ-signaling pathways. European Journal of Inflammation 11(3):615–29. doi:10.1177/1721727X1301100306.
24. Wei L, Lin J, Xu W, Cai Q, Shen A, Hong Z, et al (2012) Scutellaria barbata D. Don inhibits tumor angiogenesis via suppression of hedgehog pathway in a mouse model of colorectal cancer. International Journal of Molecular Sciences 13(8):9419–30. doi: 10.3390/ijms13089419.
25. Yadav VR, Prasad S, Sung B, Gelovani JG, Guha S, Krishnan S, et al. (2012) Boswellic acid inhibits growth and metastasis of human colorectal cancer in orthotopic mouse model by downregulating inflammatory, proliferative, invasive and angiogenic biomarkers. International Journal of Cancer 130(9):2176–84. doi: 10.1002/ijc.26251.
26. Zou DM, Brewer M, Garcia F, Feugang JM, Wang J, Zang R, et al. (2005) Cactus pear: A natural product in cancer chemoprevention. Nutrition Journal 4:25. doi: 10.1186/1475-2891-4-25.
27. Richter L, Kropp S, Proksch P, Scheu S. (2029) A mouse model-based screening platform for the identification of immune activating compounds such as natural products for novel cancer immunotherapies. Bioorganic and Medicinal Chemistry 27(23):115145. doi: 10.1016/j.bmc.2019.115145
28. Phipps HW (2016) Systematic review of traumatic brain injury animal models. Methods in Molecular Biology 1462:61–88. doi: 10.1007/978-1-4939-3816-2_5.
29. Ruggeri BA, Camp F, Miknyoczki S (2014) Animal models of disease: Pre-clinical animal models of cancer and their applications and utility in drug discovery. Biochemical Pharmacology 87(1):150–61. doi: 10.1016/j.bcp.2013.06.020.
30. De Jong M, Maina T (2010) Of mice and humans: Are they the same? -- Implications in cancer translational research. Jurnal of Nuclear Medicine 51(4):501–4. doi: 10.2967/jnumed.109.065706.
31. Schuh JCL (2004) Trials, Tribulations, and Trends in Tumor Modeling in Mice. Toxicologic Pathology 32 (Suppl 1):53–66. doi: 10.1080/01926230490424770.
32. Li Z, Zheng W, Wang H, Cheng Y, Fang Y, Wu F, et al. (2021) Application of animal models in cancer research: Recent progress and future prospects. Cancer Management and Research 13:2455–75. doi: 10.2147/CMAR.S302565.
33. Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, et al. (2010) Guidelines for the welfare and use of animals in cancer research. British Journal of Cancer 102(11):1555–77. doi:10.1038/sj.bjc.6605642.
34. Tratar UL, Horvat S, Cemazar M (2018) Transgenic mouse models in cancer research. Frontiers in Oncology 20 (8):268. doi: 10.3389/fonc.2018.00268.
35. Hanahan D, Wagner EF, Palmiter RD (2007) The origins of oncomice: A history of the first transgenic mice genetically engineered to develop cancer. Genes and Development 21(18):2258–70. doi: 10.1101/gad.1583307
36. Shah U, Shah R, Acharya S, Acharya N (2013) Novel anticancer agents from plant sources. Chinese Journal of Natural Medicine 11(1):16–23. doi:10.1016/S1875-5364(13)60002-3.
37. Khan T, Ali M, Khan A, Nisar P, Jan SA, Afridi S, et al. (2019) Anticancer plants: A review of the active phytochemicals, applications in animal models, and regulatory aspects. Biomolecules 10(1). doi: 10.3390/biom10010047.
38. Pagano JS, Blaser M, Buendia MA, Damania B, Khalili K, Raab-Traub N, et al. (2004) Infectious agents and cancer: Criteria for a causal relation. Seminars in Cancer Biology 14(6):453–71. doi: 10.1016/j.semcancer.2004.06.009.
39. Tsuda H, Ohshima Y, Nomoto H, Fujita K ichi, Matsuda E, Iigo M, et al (2004) Cancer prevention by natural compounds. Drug Metabolism and Pharmacokinetcs 19(4):245–63. doi: 10.2133/dmpk.19.245.
40. Liu Z, Brooks RS, Ciappio ED, Kim SJ, Crott JW, Bennett G, et al. (2012) Diet-induced obesity elevates colonic TNF-α in mice and is accompanied by an activation of Wnt signaling: A mechanism for obesity-associated colorectal cancer. Journal of Nutritional Biochemistry 23(10):1207–13. doi: 10.1016/j.jnutbio.2011.07.002

41. Ooi LL, Zhou H, Kalak R, Zheng Y, Conigrave AD, Seibel MJ, et al. (2010) Vitamin D deficiency promotes human breast cancer growth in a murine model of bone metastasis. Cancer Research 70(5):1835–44. doi:10.1158/0008-5472.CAN-09-3194.
42. Cheon DJ, Orsulic S (2011) Mouse models of cancer. Annual Review of Pathology 6:95–119. doi: 10.1146/annurev.pathol.3.121806.154244.
43. Henry NL, Hayes DF (2012) Cancer biomarkers. Molecular Oncology 6(2):140–6. doi: 10.1016/j.molonc.2012.01.010.
44. Srinivas PR, Kramer BS, Srivastava S (2001) Trends in biomarker research for cancer detection. The Lancet Oncology 2(11):698–704. doi: 10.1016/S1470-2045(01)00560-5.
45. Slater EP, Strauch K, Rospleszcz S, Ramaswamy A, Esposito I, Klöppel G, et al (2014) MicroRNA-196a and -196b as potential biomarkers for the early detection of familial pancreatic cancer. Translational Oncology 7(4):464–71. doi: 10.1016/j.tranon.2014.05.007.
46. Brake T, Connor JP, Petereit DG, Lambert PF (2003) Comparative Analysis of Cervical Cancer in Women and in a Human Papillomavirus-Transgenic Mouse Model: Identification of Minichromosome Maintenance Protein 7 as an Informative Biomarker for Human Cervical Cancer. Cancer Research 63(23):8173–80.
47. Lyons SK (2005) Advances in imaging mouse tumour models in vivo. The Journal of Pathology 205(2):194–205. doi: 10.1002/path.1697.
48. Li S, Chen L xiang, Peng X hua, Wang C, Qin B yin, Tan D, et al. (2018) Overview of the reporter genes and reporter mouse models. Animal Models and Experimental Medicine 1(1):29–35. doi: 10.1002/ame2.12008.
49. Lee KW, Bode AM, Dong Z (2011) Molecular targets of phytochemicals for cancer prevention. Nature Reviews Cancer 11(3):211–8. doi: 10.1038/nrc3017.
50. Navale AM (2013) ANIMAL MODELS OF CANCER: A REVIEW. International Journal of Pharmaceutical Sciences and Research 4(1):19–28.
51. Chulpanova DS, Kitaeva KV, Rutland CS, Rizvanov AA, Solovyeva VV (2020). Mouse tumor models for advanced cancer immunotherapy. International Journal of Molecular Sciences 21(11):1–15. doi: 10.3390/ijms21114118.
52. McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL (2010). Guidelines for reporting experiments involving animals: The ARRIVE guidelines. British Journal of Pharmacology 160(7):1573–6. doi: 10.1111/j.1476-5381.2010.00873.x.
53. Hollingshead MG (2008) Antitumor efficacy testing in rodents. Journal of the National Cancer Institute 2008;100(21):1500–10. doi: 10.1093/jnci/djn351
54. Cekanova M, Rathore K (2014) Animal models and therapeutic molecular targets of cancer: utility and limitations Drug Design, Development and Therapy 8:1911–22.doi: 10.2147/DDDT.S49584
55. Gayatri S, Maheswara Reddy CU, Chitra K, Parthasarathy V (2015) Assessment of in vitro cytotoxicity and in vivo antitumor activity of Sphaeranthus amaranthoides burm.f. Pharmacognosy Research 7(2):198–202. doi: 10.4103/0974-8490.150544
56. Ayers GD, McKinley ET, Zhao P, Fritz JM, Metry RE, Deal BC, et al (2010) Volume of preclinical xenograft tumors is more accurately assessed by ultrasound imaging than manual caliper measurements. Journal of Ultrasound in Medicine 29(6):891–901. doi: 10.7863/jum.2010.29.6.891
57. European Medicines Agency. The role of the pathological complete response as an endpoint in neoadjuvant breast cancer studies; 2014. Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/draft-guideline-role-pathological-complete-response-endpoint-neoadjuvant-breast-cancer-studies_en.pdf.
58. Frangioni JV (2008) New technologies for human cancer imaging. Journal of Clinical Oncology 26(24):4012–21. doi: 10.1200/JCO.2007.14.3065
59. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. (200) New guidelines to evaluate the response to treatment in solid tumors. Journal of the National Cancer Institute 92(3):205–16. doi: 10.1093/jnci/92.3.205
60. Narang AS, Desai DS (2009) Anticancer drug development: Unique aspects of pharmaceutical development. Pharmaceutical perspectives of cancer therapeutics. 2009:49-92. doi: 10.1007/978-1-4419-0131-6_2.
61. Steu S, Baucamp M, Von Dach G, Bawohl M, Dettwiler S, Storz M, et al. (2008) A procedure for tissue freezing and processing applicable to both intra-operative frozen section diagnosis and tissue banking in surgical pathology. Virchows Arch 452(3):305–12. doi: 10.1007/s00428-008-0584-y.
62. Mayeux R 2004 Biomarkers: Potential Uses and Limitations. NeuroRx 1(2):182–8. doi: 10.1602/neurorx.1.2.182.
63. McCoy CE 2017 Understanding the intention-to-treat principle in randomized controlled trials. Western Journal of Emergency Medicine 18(6):1075–8. doi: 10.5811/westjem.2017.8.35985.



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