



Therapeutic Effects of Aqueous and Ethanolic Extracts of *Phyllanthus amarus* on 1, 2 Dimethylhydrazine Induced Colon Carcinogenesis in Balb/C Mice

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Authors' contributions

This work was carried out under supervision of author SK. Author SK designed and supervised the study. Author FOO conducted the lab and animal related work, performed the statistical analysis and wrote the draft of the manuscript. Author GEE designed the protocol for plant extracts and conducted the literature searches. All authors have analyzed the results, read and approved the final manuscript.

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ABSTRACT

Context: *Phyllanthus amarus* is traditionally used for various infections, inflammation and cancer. 1,2 Dimethylhydrazine is a potent colon cancer inducer in animals.

Objective: The present study investigated the effects of aqueous and ethanolic extract of *Phyllanthus amarus* on 1, 2 Dimethylhydrazine induced colon cancer in BALB/c Mice.

Materials and Methods: 30 female Balb/C Mice of weight 18-30 g were acclimatized for a week and randomized into 6 groups (5 per group). Group A (-DMH), Group B (+DMH), Group C (DMH+250 mg/kg body weight of ethanolic extract of *P. amarus*), Group D (DMH+350 mg/kg body weight of ethanolic of *P. amarus*), Group E (DMH + 250 mg/kg body weight of aqueous extract of *P. amarus*), Group F (DMH+ 350 mg/kg body weight of aqueous extract of *P. amarus*). 20 mg/kg body weight of DMH was administered orally for 21 days (twice a week). The plant extracts were

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administered daily for 3 weeks with the aid of a gavage immediately after colon cancer induction. Colon cancer was evaluated by the formation of Aberrant Cryptic Foci in the colon of DMH treated mice.

Results: Administration of the plant extracts (aqueous and ethanolic) ameliorated the carcinogenic effect of DMH in the colon of DMH treated mice in a dose dependent manner by significantly reducing the number of Aberrant Cryptic Foci formed in extract treated mice by 38% for 350 mg/kg body of ethanolic extract and by 22% for 350 mg/kg body of aqueous extract of *Phyllanthus amarus*.

Conclusion: The studied extracts had ameliorative potential on DMH induced colon cancer in Balb/C mice in a dose dependent manner providing evidence for the traditional use of this herb for treatment/prevention of cancer. Notably, 350 mg/kg body of both extracts showed better reduction of Aberrant Cryptic Foci compared to 250 mg/kg body of both ethanolic and aqueous extracts of *Phyllanthus amarus*.

Keywords: DMH (Dimethylhydrazine); *Phyllanthus amarus*; Aberrant Cryptic Foci (ACF); carcinogen.

1. INTRODUCTION

The term medicinal plant includes a variety of plants used in herbalism and some of these plants possess medicinal properties. Medicinal plants are considered a rich source of ingredients which can be used in drug development and synthesis hence they are recommended for their therapeutic value [1]. The Genus *Phyllanthus* (family: *Phyllanthaceae*) consists of approximately 1000 species, spread over the American, African, Australian and Asian Continents [2]. *Phyllanthus amarus* is one of the most pharmacologically important species of the *Phyllanthus* family. It is a medicinally important plant belonging to Euphorbiaceae otherwise known as “stone breaker”, “carry me seed” etc. *Phyllanthus amarus* is an erect annual herb of not more than one and half feet tall. It has small leaves and yellow flowers. It is commonly found in forest areas, arid land, savannah areas, leached and exhausted soil in many countries including China, India, Nigeria, Cuba and Philippines amongst others [3,4]. Most of the herbs, belonging to the *Phyllanthus* family, afford various secondary metabolites with important medicinal properties. Bioactives such as alkaloids, flavonoids, lignin, phenols, tannins and terpenes have been isolated from these plants [5,6]. Due to its impressive preclinical therapeutic properties, extracts of species of the *Phyllanthus* have been evaluated to treat hypertension, jaundice, and diabetes. Other studies revealed preclinical pharmacological activity and therapeutic potential of phytochemicals isolated from *Phyllanthus amarus* [7]. The powdered leaves of *Phyllanthus amarus* (Bahupatra) were used in clinical studies evaluating its usefulness in patients suffering from chronic damage to the liver due to protracted hepatitis B virus infection. Based on its very

useful medicinal properties, *Phyllanthus amarus* is very frequently utilized in traditional medicine [8].

Cancer is a large group of disorders characterized by uncontrolled cellular proliferation. Cancer cells are also capable of metastasizing to other regions causing a number of devastating outcomes [9]. Nearly all body organs are vulnerable to cancer with liver, colon, and breast being the most common ones. Colon cancer is a type of cancer that begins in the large intestine (colon). The colon is the final part of the digestive tract. Colon cancer has an estimated incidence of over 1 million new cases annually worldwide [10]. Almost one of three patients with colon cancer dies from the disease. Colon cancer also more often affects people of well-developed countries in comparison to less developed countries [11]. Colorectal cancer is one of the leading causes of tumor-related death and despite its high prevalence, the underlying pathological mechanism remain elusive [9]. Colorectal cancer is a multistep process affected by environmental and genetic factors which lead to normal colonic epithelium to dysplasia followed by a benign precursor stage, the pre-malignant polyp and can progress to invasive disease. Besides a genetic pre-disposition, diet also determines the risk for colon cancer and predominantly diets rich in fruit and vegetable diminish the risk of the disease [10].

1,2 Dimethylhydrazine -induced model was utilized, as it is similar to histopathological and molecular characteristics of the human colon cancer model [11]. 1,2 Dimethylhydrazine is metabolized in the liver to form Azoxymethane and methylazoxymethanol later transported to the colon via bile or blood to generate its ultimate carcinogenic metabolite, diazoniumion which

elicits oxidative stress by methylating biomolecules of the colonic epithelial cells, leading to promutagenic events as a result of inflammation and tumors in the colon [12].

Recently, interest in the search of naturally occurring antioxidants from plants has been rekindled. This research work investigated the ameliorative ability of aqueous and ethanolic extracts of *Phyllanthus amarus* leaves on 1,2 Dimethylhydrazine induced colon carcinogenesis in mice model.

1.1 Aim of Study

This study aims to evaluate the therapeutic effects of aqueous and ethanolic extracts of *Phyllanthus amarus* leaves on 1,2 Dimethylhydrazine-induced colon cancer in Balb/C mice.

2. MATERIALS AND METHODS

2.1 Plant Collection

The leaves of *Phyllanthus amarus* were collected from the botanical garden of University of Benin, Nigeria and were identified by a Botanist in the Department of Plant Biology and Biotechnology, University of Benin, Benin City.

2.2 Plant Sample Preparation

The leaves of this plant were air-dried in the laboratory at the Department of Biochemistry, University of Benin, Benin City. The leaves were later pulverized to powdery form in Pharmacognosis laboratory at the Faculty of Pharmacy, University of Benin. 250 g of the powdered leaves of *Phyllanthus amarus* was soaked in 1.5 liters of absolute ethanol for 24 hours with periodic stirring of the mixture. After 24 hours, the mixture was filtered with fine cheese cloth, the residue was discarded and the filtrate was used to soak another 250 g of powdered leaves of same plant above, allowed to stand for another 24 hours with continuous stirring, thereafter, the mixture was again filtered. The residue was discarded and the filtrate was filtered with whatman filter paper (No: 1) and was concentrated with the aid of a vacuum concentrator at 30°C. The concentrates were then weighed and used as experiment sample. The same procedure was carried out with distilled water for preparing the aqueous extract. The above isolation of crude extract was done at the Department of Biological Sciences, Birla

Institution of Technology and Science, BITS-Pilani, Hyderabad, India.

2.3 Phytochemical Screening

Phytochemical screening to detect the presence of bioactive agents was performed by standard procedures [13,14]. After the addition of specific reagents to the solution, the tests were detected by visual observation of color change or by precipitate formation.

Chemical: DMH was purchased from TCI Chemical, Chennai.

Animal Study: 30 female Balb/C Mice of weight 18-30 g were purchased from VAB Biosciences, Bapuji Nagar, Musheerabad, Hyderabad-500020. They were maintained according to the Institutional Animal Ethics Guidelines (1912/PO/Re/S/16/CPCSEA) and acclimatized to diet and environment for 1 week after arrival. They were housed 5 animals per rack mounted plastic with detachable steel aerated covered cages and were given clean drinking water ad libitum. The temperature (20-22°C) and lighting (12 hours light/dark cycle) were constantly controlled. DMH was dissolved in Millipore water and was administered (20 mg/kg body) orally. The volumes of gavage were from 0.18-0.3 ml. Oral administration of DMH lasted for 21 days (twice a week). Upon completion of the doses of carcinogen, the DMH treated mice were randomized in 5 groups, groups B – F. Group B served as positive control, Group C received 250 mg/kg body ethanolic extract of *P. amarus*, Group D received 350 mg/kg body weight of ethanolic extract of *P. amarus*, Group E received 250 mg/kg body weight of aqueous extract of *P. amarus* and Group F received 350 mg/kg body weight of aqueous extract of *P. amarus*. The plant extracts were administered daily as an oral gavage for 21 days.

Analysis of Aberrant Cryptic Foci: Aberrant Cryptic Foci (ACF) were analyzed at the end of the experiment using procedure described by Barbara et al. [15]. The animals were killed by cervical dislocation and their colons were removed and flushed with Kreb's ringer salt solution. The colons were cut open along the longitudinal axis and fixed flat between filter paper in 10% buffered-formalin solution for 24 hours and were stained with Methylene blue (0.05% in Kreb's ringer salt solution) for 30 minutes in order to visualize crypts' outlines. The colons were mounted on microscopic slides with the mucosal surface up and aberrant crypts were

scored under a confocal microscope at a magnification of 20 x. The number and location of the aberrant crypts were recorded. Aberrant crypts were distinguished from surrounding normal-appearing crypts based on 3 characteristics; increased size, significantly increased distance from the luminal to basal surfaces of cells, and the easily discernible pericryptal zone.

2.4 Statistical Analysis

Counts of ACFs were expressed as means ± SEM. All statistics were computed using a suitable program (Microsoft Excel & Graph pad prism 7). Values of $P < 0.05$ was considered significant.

3. RESULTS

In this study, the preventive effects of aqueous and ethanolic extract of *Phyllanthus amarus* was investigated for their anti-colon cancer ability in Balb/C mice. It holds an array of secondary metabolites with medically important properties.

Table 1. Phytochemical screening

Phytochemicals	Ethanol	Aqueous
Tannin	+	+
Saponin	+	+
Flavonoids	+	+
Glycosides	-	+
Quinones	+	+
Phenols	+	+
Terpenoids	+	-
Steroids	+	-

Key: + for present and –for absent

4. DISCUSSION

Plants bioactives have the potential to be major chemo-protective ingredients to control cancer. Substantial evidence indicates that plant bioactives may play an essential role in colon cancer prevention and management [16]. In this study, the preventive effects of aqueous and ethanolic extract of *Phyllanthus amarus* was investigated for their anti-colon cancer ability in Balb/C mice. It holds an array of secondary metabolites with medically important properties. Bioactives such as alkaloids, flavonoids, lignin, phenols, tannins and terpenes have been isolated from this plant [5,6]. The results from this study show that *P.amarus* has preventive properties against colon cancer induced by DMH. Its medical properties could be due to the presence of useful phytochemicals and antioxidant such as flavonoids, polyphenols, alkaloids and tannin. They are major contributor to anticancer and anti-hepatotoxic capability of most medicinal plants [17]. Numerous epidemiological studies have validated the inverse relation between the consumption of flavonoids and the risk of cancer. Flavonoids possess cancer blocking and suppressing effects, they are involved in the regulation of enzymes of phase- II responsible for xenobiotic biotransformation and colon microflora [18]. Colon cancer is a major cause of morbidity and mortality throughout the world [19]. It accounts for over 9% of all cancer incidences [20,21]. It is the third most common cancer worldwide and the fourth most common cause of death [21]. It affects men and women almost equally, with

Table 2. Data showing the weight of animals

Weights (g)	Group A	Group B	Group C, DMH	Group D, DMH	Group E, DMH	Group F, DMH
	(-ve cont.)	(+ve cont.)	250 mg/kgbw. Et. <i>P.a</i>	350 mg/kgbw. Et. <i>P.a</i>	250 mg/kgbw. Aq. <i>P.a</i>	350 mg/kgbw. Aq. <i>P.a</i>
Initial wt.	31.30±0.82	29.80±1.01	19.03±0.75	19.47±0.81	18.82±0.82	19.03±0.32
Final wt.	40.47±2.20	31.67±2.85	21.93±1.09	21.71±1.86	21.66±3.67	22.97±1.21
Weight gain	9.21	1.87	2.92	2.24	2.84	3.94

Table 3. Data showing number of aberrant cryptic foci in the colons of experimental animals

Groups	Treatment	Number of ACF	Number of Crypts/focus
Group A	-DMH	—	—
Group B	+DMH	21.67±4.71	1
Group C	DMH+250 mg/kgbw.(Etoh of <i>P.amarus</i>)	19.50±2.04	1
Group D	DMH+350 mg/kgbw.(Etoh of <i>P.amarus</i>)	13.33±2.33*	1
Group E	DMH+250 mg/kgbw.(Aq of <i>P.amarus</i>)	19.00±1.01*	1
Group F	DMH+350 mg/kgbw (Aq of <i>P.amarus</i>)	16.66±2.03*	1

over 1.8 million new cases recorded in 2018, the most recent year for which international estimates are available [19,20,22,23,24]. Countries with the highest incidence rates include Australia, New Zealand, Canada, the United States, and parts of Europe. The countries with the lowest risk include China, India, and parts of Africa and South America [20]. Aberrant cryptic foci are useful intermediate

biomarkers in detecting modifying influences of natural and synthetic compounds on chemically induced colon carcinogenesis, which represents the preneoplastic lesions [25]. Cell proliferation, plays an important role in multistage carcinogenesis with multiple genetic changes [26]. Modulation of cell-proliferation activity in target organs is one of the important actions of cancer chemoprevention [27].

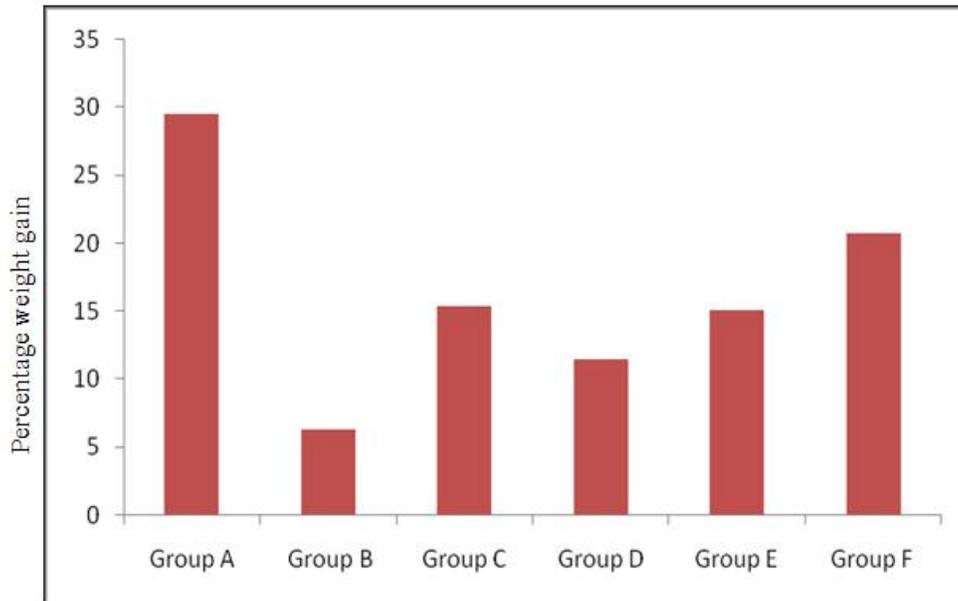


Fig. 1. Showing percentage weight gain in experimental animal

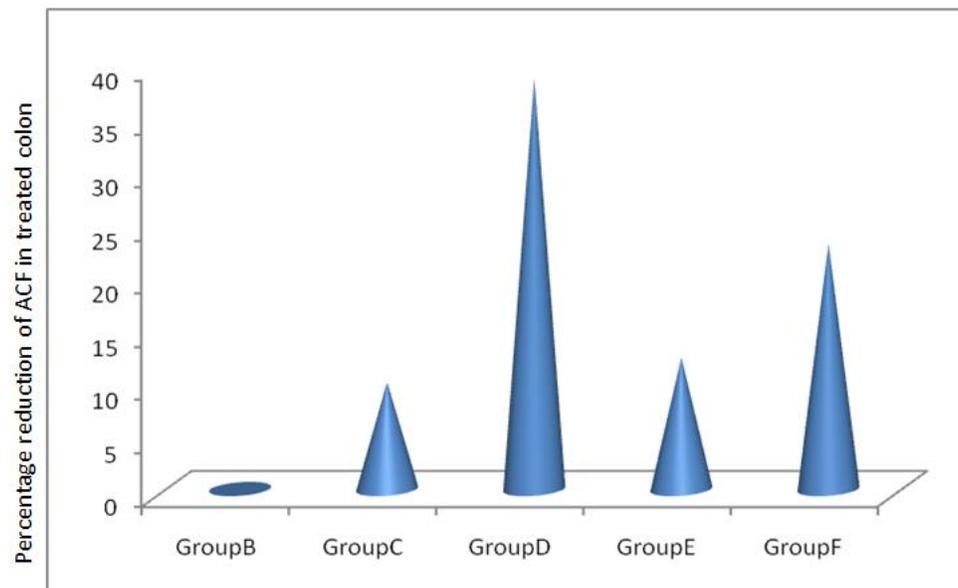


Fig. 2. Showing percentage reduction of ACF in extract treated colon

Table 4. Data showing number of aberrant crypt foci/ location of mice colon

Groups	Proximal	Mid	Distal
Group A (-DMH)	--	--	--
Group B (+DMH)	6.67±1.33	4.67±1.77	10.33±3.69
Group C (DMH, 250 mh/kgbw Et. <i>P. amarus</i>)	1.00±0.82	6.00±1.63	12.50±2.86
Group D (DMH, 350 mh/kgbw Et. <i>P. amarus</i>)	7.00±2.08	2.00±0.99	4.33±2.03
Group E (DMH, 250 mh/kgbw Aq. <i>P. amarus</i>)	1.50±0.50	9.00±1.01	8.50±0.70
Group F (DMH, 350 mh/kgbw Aq. <i>P. amarus</i>)	3.33±0.33	7.33±1.20	6.00±0.58

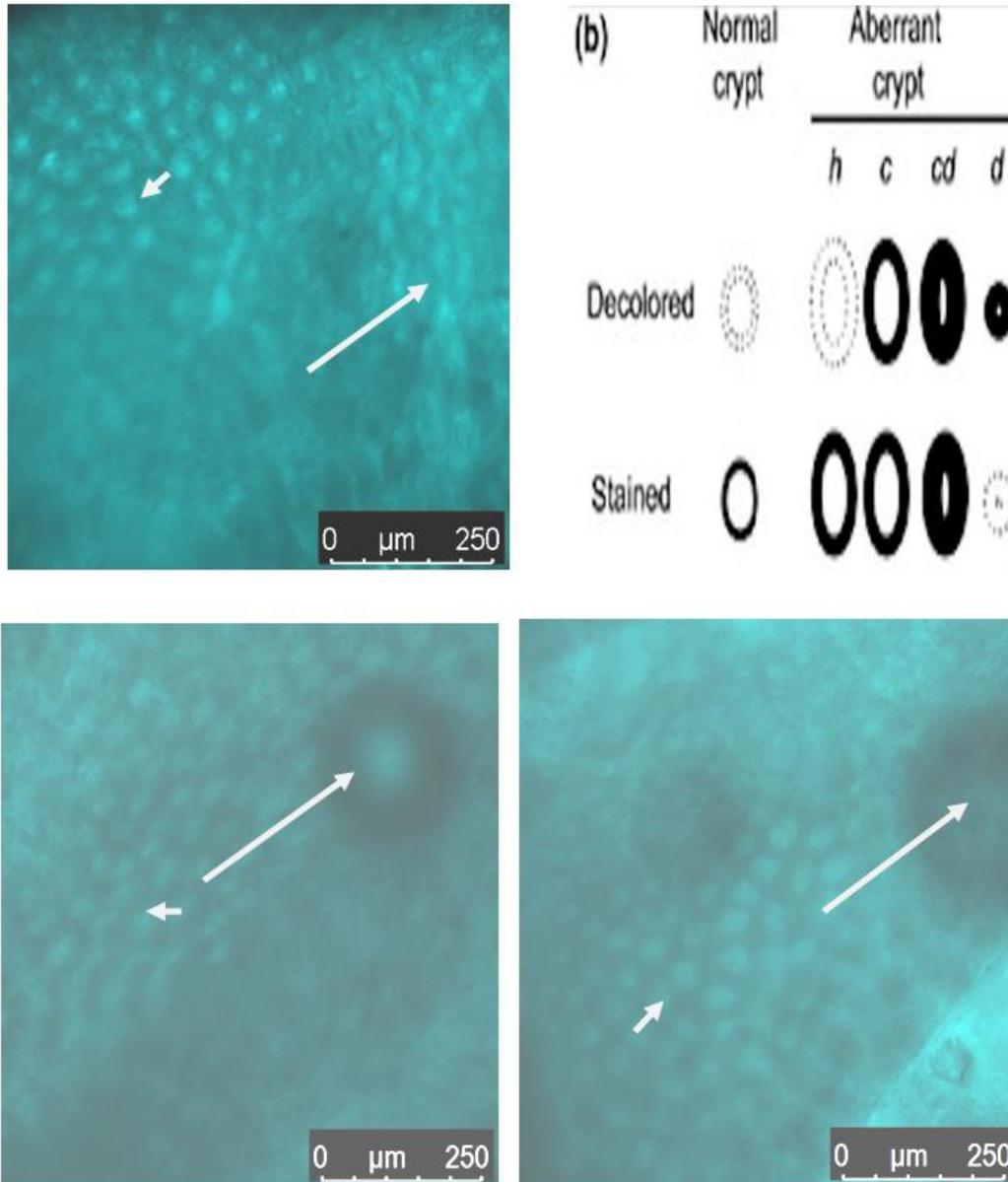


Fig. 3. Showing microscopic view of mice colon with ACF at a magnification of 20x under confocal microscope. (b) Schematic view of the crypt subtypes; (c)-type (enlarged crypt and broad opening); (c, d)-type enlarged crypt and narrow opening; d-type small crypt and narrow opening. A ring-shaped image represents the morphology of epithelial lining [31]

In the current study, DMH induced aberrant cryptic foci in all the groups treated with the cocktail (DMH) after 3 weeks of administration. This effect was significantly reduced in a dose dependent manner as observed in the groups treated with different doses of the extract as summarized in Tables 3 and 4. Ethanolic extract at concentration 350 mg/kg body weight had a more significant reduction on ACF formation in the colon than its counterpart (aqueous extract) at the same concentration. It is noteworthy that treatment with both extracts at 350 mg/kg body weight significantly reduced ACF formation. Though there was mild reduction in colon treated with 250 mg/kg body weight, but the difference was insignificant. Some effect of *P. amarus* was also seen on the weight of the experimental animals. Groups treated with the plant extracts had increase weight relatively close to negative control (-DMH) group, but significantly higher than the weight of positive control (+DMH) group. This could be due to the phytonutrients (vitamins and minerals) present in the plant extract. The preventive effect of *P. amarus* in colon cancer could also be linked to the secondary metabolite, phenolic acid. Phenolic acid is implicated for its ant-proliferative and pro-apoptotic effects in colon cancer cell line in a concentration dependent manner [28]. Lignin, a bioactive in *P. amarus* also contributes to the prevention of colon cancer. The mechanism of action is the ability of colon bacteria to convert it into biologically active lignans such as enterodiol and enterolactone. These lignans are structurally similar to estradiol and therefore, they exert anticancer effects on hormone-related cancer [29,30].

5. CONCLUSION

This preliminary study was to investigate the preventive effects of aqueous and ethanolic extracts of *Phyllanthus amarus* on 1, 2 Dimethylhydrazine induced colon carcinogenesis in Balb/C mice. The overall results suggest that the plants extract had preventive effects on DMH induced colon carcinogenesis in Balb/C mice. Notably, 350 mg/kg body weight of both extracts showed higher bioactivity than 250 mg/kg body weight and this bioactivity is higher in ethanolic extract in comparison to the aqueous extract at the same concentration.

DISCLAIMER

The products used for this research are commonly and predominantly used products in

our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

Animal Ethics committee approval has been taken to carry out this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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