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EVALUATION OF THE ANTIPROLIFERATIVE AND ANTIMITOTIC EEFECTS OF WATER EXTRACT OF *Piper nigrum* SEEDS USING THE *Allium cepa* ASSAY.

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ABSTRACT

Background: Many bioactive substances in plants have the potentials to affect physiological processes in both plants and animals. One of the most common tests used in the evaluation of antimitotic and antiproliferation properties of plant extracts is the *Allium cepa* assay.

Aim:

This study was aimed to screen the seeds of *P. nigrum*, a common spice used in Cameroonian cuisines, for antiproliferative and antimitotic properties to and hence confirm their anticancer activity.

Materials and Methods:

Onion bulbs were treated to various concentration of the water extracts of dried seeds of *P. nigrum* for 72 hours. To determine the antiproliferative and antimitotic effects of the extracts, the onion bulbs were examined for sprouting of new roots, growth in length of the roots, mitotic indices and chromosomal abnormalities.

Results and conclusion:

The extracts were antiproliferative and antimitotic to the roots of *A. cepa*. The number of roots sprouted, the length of the roots and mitotic index (MI) significantly decreased with increase in concentration of the extract. Chromosomal aberrations such as chromosome bridges, chromosome laggards, chromosome stickiness and vagrants were recorded indicating the cytogenotoxic nature of the extracts.

Key words: Antiproliferative, Antimitotic, water extract, P. nigrum seeds, allium cepa.

INTRODUCTION

Piper nigrum is one of the two flowering decorous climbing vines in the Piperaceae that is native to India, Sri Lanka, and tropical regions. In Cameroon, the dry seeds of *P. nigrum* variously referred to as black pepper, bush pepper and kontri pepper is a common spice used in almost all cuisines and form an important component of soups used to spice grilled fish, chicken and meat. In addition, *P. nigrum* seeds are used in several traditional medications for the relief of pains, rheumatism, chills, flu, colds, muscular aches and fever. Externally, *P. nigrum* is used to relax sore throat and some skin disorders. The seeds of *P. nigrum* have antimicrobial, antimutagenic, antioxidant and scavenging movement (Dorman and Dean, 2000; El-Hamss et al, 2003; Gulcin, 2005). Inhalation of the oil from *P. nigrum* is known to increase reflex swallowing movements (Vijayakumar et al, 2004). Extracts from *P. nigrum* have also been shown to have insecticidal

activities against 4th instars of *Aedes aegypti* and *Anopheles stephensi* (Siddiqui et al, 2006).

Recent research has it that *P. nigrum* is bestowed with many bioactive compounds of pharmacological significance hence the dominant piperine (alkaloid) as well as complex mixtures of monoterpenes and sesquiterpenes such as β-caryophyllene, β-thujene steroids, tannins, phenols and flavones. Some 5 phenolic amides that possess antioxidant properties have also been identified (Nakatani et al, 1986; Dodson et al, 2000; Nahak and Sahu, 2011; Vijayalaxmi, 2012; Ganesh et al, 2014; Andriana et al, 2019). The piperamides present P. nigrum extract have been reported to cause oxidative damage to in DNA leading to cell cycle arrest and apoptosis in cancer cells (Santos et al, 2016).

In spite of its pharmacological importance, the cytogenotoxic properties of *P. nigrum* seeds are yet to be assessed. This study was therefore designed to screen water extracts of *P. nigrum* seeds for antiproliferative and antimitotic activities and hence confirm the presence of anticancer properties of the extract.

MATERIAL AND METHODS

Plant materials

Dry seeds of *Piper nigrum* (Fig. 1a) were bought in the Bamenda main market located in the Northwest Region of Cameroon. The seeds with voucher specimen, *UBa/PLS-Herb/122-22* were authenticated by Dr. Tacham Walters and deposited in the Herbarium of the Department of Plant Science, Faculty of Science, The University of Bamenda.

Preparation of crude aqueous extracts

The 100 seeds of *P. nigrum* were selected to remove debris, pulverized using 500 watts blender to obtained a coarse powder. The powder obtained was stored in a labelled stoppered plastic bottle from which various amounts were taken and extracted in distilled water.

The water extracts of *Piper nigrum* were prepared according to the procedure described by Seino et al (2022) with modifications. One hundred (100) grams of powder was weighed into a conical flask to which one (1) litre of distilled water was added and left for 48 hours while stirring at intermittent intervals. The filtrate obtained formed the stock solution from which several concentrations were prepared and used for this study. Five concentrations (0µg/ml, 10µg/ml, 20µg/ml, 30µg/ml and 40µg/ml) were prepared from the stock and were used to study the antimitotic and antiproliferation effects of the extract on the meristematic cells of the root tip of onion (*Allium cepa*). Distilled water (0µgm/ml) was used as the control.

Allium cepa test

Thirty (30) onion bulbs were obtained commercially from the Bamenda central market located in the North West Region of Cameroon. The white variety imported from the north of Cameroon was used for the study. Dry onion bulbs were used in the modified *A. cepa* assay (Fiskesjö 1997; Bakare and Wale-Adeyemo 2004) to evaluate the antimitotic and antiproliferative effects of the water extracts of *P. nigrum*. Average size onion (*Allium cepa*) bulbs (about 2.0cm diameter) were cleaned by carefully removing the outer dry scales and the dry roots by scraping away the bottom of the onion without destroying the root primordia.

The experiment was set up putting about 100ml of each concentration of the extract in small transparent disposable cups. The onion bulbs were then placed in the cups such that only the bottom with the primordia touched the extracts. For each concentration and extract, five replications were prepared. The preparations were then incubated in the open laboratory (25ºC) for 72hours.

Evaluation of antiproliferative activity of the water extract of *P. nigrum* seeds

The parametres used to measure the antiproliferative effects of the water extracts of *P. nigrum* seeds included counting the number of roots sprouted and measuring the lengths of the roots at the end of the experiment. The number of primary root emissions for each onion bulb in each concentration of extract were counted manually while the lengths of roots were measured with aid of a transparent ruler. Ten (10) roots were randomly selected from each of the treated onion bulbs and measured. Therefore, the lengths of fifty (50) roots were measured per concentration of the water extract of *P. nigrum* seed.

The percentage inhibition of root sprouting was calculated using the formula (Gupta and Patel, 2020):

$$\textit{Root sprouting inhibition (\%)} = \frac{\textit{Mean No of roots in control} - \textit{Mean No of roots in treated group}}{\textit{Mean No of roots in control}} \boldsymbol{X\ 100}$$

The percentage inhibition in root growth was calculated using the formula:

$$\textit{Root growth inhibition (\%)} = \frac{\textit{Mean root lenth in control} - \textit{Mean root length in treated group}}{\textit{Mean root length in control}} \boldsymbol{X\ 100}$$

Evaluation of the antimitotic effect of the water extract of *P. nigrum* seeds.

Antimitotic activity was determined using the *A. cepa* root tip model as per the method of Gupta and Patel. (2020). The antimitotic effects of the extracts were measured through the determination of Mitotic Index (MI) and chromosome abnormalities induced in the mitotic process of the meristematic cells of the root tips of onion (*Allium cepa*). Mitotic chromosome smears were prepared from the meristematic tips of the treated roots whose lengths were measured. The chromosome smears were prepared with slight modification of the method of Parmar et al, (2021). Root tips 1cm were cut and placed in a petri dish. They were fixed in 1:3 acetic – ethanol fixative for 12hours at laboratory temperature (25 – 30oC), dehydrated with 1NHCl at 60ºC for 10 minutes, stained with acetic orcein for 15 minutes and then squashed between the thumb and flat surface of the laboratory table. The smears thus prepared were examined using the 40X objective of a Fisher bright field compound microscope for chromosome abnormalities and pictures taken with the help of an Itel A51 phone mounted with a 5.0 pixa lens. At least 500 cells were scored per treatment and control to study the mitotic index and chromosome abnormalities.

Percentage Mitotic Index (MI) were calculated using the formula (Cree et al, 2021):

$$\textit{Mitotic index (MI) (\%)} = \frac{\textit{Number of dividing cells recorded}}{\textit{Total number of cells examined}} X\ 100$$

Percentage Mitotic Inhibition were calculated using the formula:

$$\textit{Mitotic inhibition (\%)} = \frac{\textit{MI in control} - \textit{MI in treated group}}{\textit{MI in control}} X\ 100$$

The data obtained from the studies were presented as mean ± SEM and analysed using a one-way analysis of variance (ANOVA) with 'p' value less than 0.05 considered as statistically significant. The difference between the control and the treated groups in relation to root number and root length was analysed using the Student t-test.

Results and Discussion

Many bioactive substances in plants have the potentials to affect physiological processes in both plants and animals. One of the most common tests used in the evaluation of antiproliferation and antimitotic properties of plant extracts is the *Allium cepa* assay. The *A. cepa* root tip assay is actually the most common method for testing compounds with antimitotic activity. In this study, the toxic effect of *P. nigrum* seed extracts was evaluated by analysing the number of roots sprouted, the length of root tip growth, mitotic index and chromosome aberrations recorded.

Antiproliferation activity of the water extract of P. nigrum seeds

To investigate the possible growth inhibition (antiproliferation) effects of the aqueous extracts of *P. nigrum* seeds, the extracts were tested for their capability to inhibit sprouting of the roots and increase in the lengths of sprouted of *Allium cepa* roots. The effect of different concentrations of *P. nigrum* seed extracts on the number of roots sprouted and the growth in length of the roots *A. cepa* obtained during this study is presented in Fig 1 and Table 1.

These results show that all tested concentrations of *P. nigrum* seed water extracts caused significant inhibition ($p<0.05$) in the sprouting and growth in length of roots as compared to the control. The highest activity was recorded for the extract with concentration 40 µg/ml (85.17% and 83.18% for sprouting of roots and length of roots respectively).

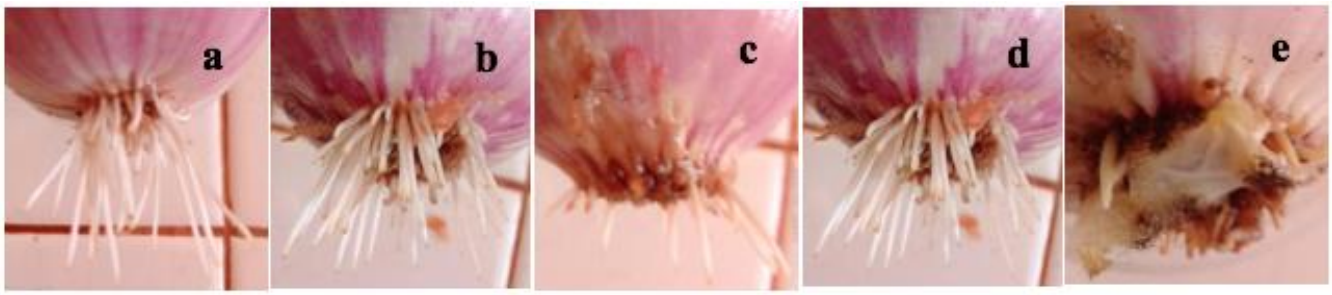

Fig. 1: Pictures of Onion (*Allium cepa*) roots to show the effect of different concentrations of the water extract of the seeds of *Piper nigrum*: a) Control (0.0 µg/ml) b) 10.0 µg/ml, c) 20.0 µg/ml, d) 30.0 µg/ml and e) 40 µg/ml.

Table 1.: Mean number of roots sprouted and length of roots of *Allium cepa* obtained on treatment with various concentrations of aqueous extracts of the seeds of *Piper nigrum*.

Concentration (µg/ml)	Mean number of roots sprouted	Mean length of roots (cm)	% inhibition of root sprouting	% inhibition of root growth in length
Control (distilled H_2O)	70.8 ± 0.52^a	4.34 ± 0.07^a	0.0^a	0.0^a
10	51.2 ± 0.46^b	1.64 ± 0.08^b	27.68^b	62.21^b
20	36.0 ± 0.33^c	0.98 ± 0.11^c	49.15^c	77.42^c
30	17.2 ± 0.12^d	0.96 ± 0.05^c	75.71^d	77.88^c
40	10.5 ± 1.6^e	0.73 ± 0.08^c	85.17^e	83.18^c

Number of trials n=5, $p<0.05$; significantly different from control group (Distilled water), applying one-way ANOVA followed by Tukey's post-test (HSD). Groups that have no letters in common differ significantly.

The lowest activity (27.68% and 62.21% for sprouting of roots and length of roots respectively) was recorded for the extract at 10 µg/ml concentration. However, inhibition was more severe for root sprouting than growth in root length. The inhibition increased significantly ($p<0.05$) with increase in concentration of the extract. In spite of this, percentage inhibition of root sprouting and root length increased concentrations of *P. nigrum* seed extracts.

Antimitotic assay and mitotic index (MI)

Antimitotic agents are the compounds that arrest cells multiplication in mitosis. They can interrupt the process of mitosis during any phase of the cell cycle. This section of the study was designed to investigate the possible mechanism involved in root growth inhibition. Hence, the antimitotic activity of the water extracts of *P. nigrum* were determined by analysis of cytological parametres like Mitotic Index (MI) and the chromosomal abnormalities. The chromosomal abnormalities investigated included laggards, bridges, stickiness and disoriented anaphase chromosomes (vagrants). The results obtained in this study revealed differences in mitotic index in all concentrations of *P. nigrum* seed extract tested (Table 2). The highest mitotic index (MI) (6.3%) was recorded in the control (0 µg/ml) while the lowest mitotic index (0.9%) was recorded at the 40 µg/ml concentration of the *P. nigrum* extract. The mitotic index (MI) of *A. cepa* meristematic root tip cells treated with water extract of *P. nigrum* was therefore significantly decreased (0.9 %) in comparison to the control (6.3%). The mitotic index was positively correlated with increasing concentration of the *P. nigrum seed* extracts. (Table 2).

Table 2.: Effect of concentration of *P. nigrum* extract on Mitotic index (MI) of *Allium cepa* cells.

Concentration (µg/ml)	N0 of dividing cells	Mitotic Index (MI)	Mitotic inhibition
Control (distilled H_2O	63	6.3^a	0.0^a
10	52	5.2^b	17.5^b
20	36	3.6^c	42.9^c
30	19	1.9^d	69.8^d
40	9	0.9^e	85.7^e

Number of trials n=5, $p<0.05$; significantly different from control group (Distilled water), applying one-way ANOVA followed by Tukey's post-test (HSD). Groups that have no letters in common differ significantly.

Evidences abound in literature that the cytotoxicity of plant extracts affect mitotic index (MI). Also, increases in plant extract concentration often result in decreases of mitotic index (MI) (Akinboro and Bakare 2007, Oyeyemi and Bakare 2013, Chukujekwu and Van Staden, 2014; Owolarafe et al., 2020). The results obtained in this study on the effect of different concentrations of the water extracts of *P. nigrum* confirmed that the seeds of *P. nigrum* contain antimitotic agents (compounds that can arrest cell multiplication in mitosis). The decrease MI in *A. cepa* roots treated with water extracts of *P. nigrum* could be explained by the prevention of DNA synthesis in the S-phase of the cell cycle and the decrease in the number of cells that are dividing in the roots as a result of the cytotoxic effects of bioactive compounds

found in *P. nigrum* seeds. Also, some of the phytochemicals in the plant extract could also interact with the formation of the spindle apparatus in the meristematic root tip cells of *A. cepa* thus reducing mitotic index (MI) (Raheel et al, 2017). During such interaction, the mitotic apparatus of the cell is blocked and the transition from interphase to the mitotic phase of the cell cycle fails to take place. It therefore follows that low mitotic index is the result of a direct genotoxic effect of the water extract of the seeds of *P. nigrum* on the cells of *A. cepa*. Since mitosis was affected, it is but normal that sprouting of roots as well as growth in length of the roots had to be slowed down.

Chromosomal abnormalities

The aqueous extract of *P. nigrum* showed very strong genotoxic effects in the meristematic cells of *A. cepa* root tips. Chromosomal analysis revealed aberrations such as anaphase laggards and bridges, metaphase stickies and disoriented anaphase chromosomes (vagrants) (Fig. 2 and Table 3).
Table 3 revealed that *P. nigrum* seed extracts induced chromosomal alterations and there was an increase in the number of abnormalities with increase in concentration of extract. The toxic effects recorded were however not great as shown by the low percentage occurrences of sticky metaphases. The mitotic inhibition was high with higher concentrations of *P. nigrum* extract (40µg/ml). The absence of chromosome fragments could probably be the reason there was no cellular death (Celik and Aslanturk, 2010).

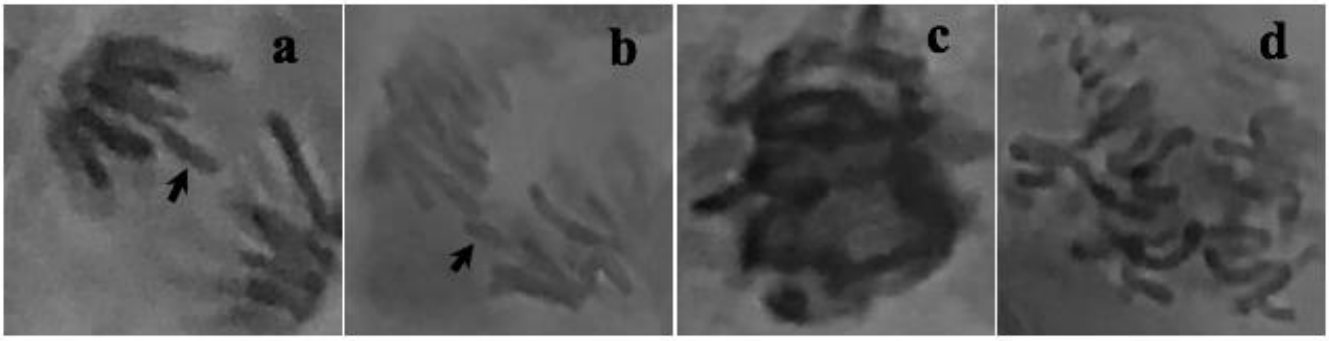

Fig.2.: Some chromosomal abnormalities induced in *A. cepa* meristematic root cells, by water extracts of the seeds of *P. nigrum*. a) Chromosome laggards, b) Anaphase bridges, c) Sticky, d) Disoriented Anaphase (vagrants).

Table 3: Chromosome and mitotic aberrations in the root meristem cells of *Allium cepa* after extract treatment with various concentrations of aqueous extract of *P.nigrum*.

Concentration of extract	Chromosome bridges (%) ±SD	Chromosome laggards (%) ±SD	Sticky chromosomes (%) ±SD	Anaphase vagrants (%) ±SD	Total chromosome aberrations (%) ±SD
Distilled H_2O	-	-	-	-	-
10µg/ml	-	0.19 ± 1.05	0.27 ± 1.67	-	0.46 ± 2.55[a]
20µg/ml	0.18 ± 1.33	0.37 ± 3.12	0.75 ± 0.77	-	1.30 ± 3.64[b]
30µg/ml	0.27 ± 2.12	0.54 ± 2.32	0.22 ± 3.52	0.26 ± 3.28	2.59 ± 3.56[c]
40µg/ml	0.39 ± 2.33	1.42 ± 3.66	1.06 ± 4 .05	0.21 ± 0.91	3.08 ± 3.49[d]

Number of trials n=5, $p<0.05$; significantly different from control group (Distilled water), applying one-way ANOVA followed by Tukey's post-test (HSD). Groups that have no letters in common differ significantly.

The presence of chromosomal abnormalities could be due to the fact that active bioactive substances in the extract of *P. nigrum* affected microtubule formation (Mitchison, 2012, Raheel et al. 2017) and DNA synthesis (Celik and Aslanturk, 2010). Further, the bioactive substances in the *P. nigrum* seed extract could also induce disturbances in microtubule formation leading to the formation of anaphase bridges (Swierenga et al, 1991).

Though the primary aim of this study was not to determine the anticancer activity of the water extract of the seeds of *P. nigrum*, it is however worthy to note that the mitosis in A. *cepa* root tip cells is similar to cell division in normal human and cancer cells. For this reason, meristematic root tip cells of A. cepa can be used to test drugs that have anticancer activity (Saboo et al., 2007). This study has therefore revealed that the bioactive components of *P. nigrum* have

beneficial health effects and can be used as a possible therapeutic agent against cancer.

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AUTHORS CONTRIBUTION

- SRA conceived the research
- SRA, NA & DTI conducted the research experiments as well as carried out the statistical analysis.
- SRA wrote the manuscript.

DECLARATION ON CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

All experimental studies on plants have complied with relevant institutional, national and international guidelines and legislation.