Journal Of Biomedical Investigation (JBI) Vol. 12 no. 1 April-May 2024

Curcuma longa AND *Moringa oleifera* ARE SYNERGISTICALLY ANTIPROLIFERATIVE BY DOWNREGULATING p63 GENE IN TESTOSTERONE-INDUCED BENIGN PROSTATE HYPERPLASIA IN RATS

Authors:
OKOYE, Jude Ogechukwu[1,2*]; AKINYEMI, Taiwo Elizabeth [2]; MADUEKWE, Jonathan[3]

Authors Affiliations:
[1]Department of Medical Laboratory Science, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, Nigeria.
[2]Department of Medical Laboratory Science, School of Public and Allied Health, Babcock University, Ogun State, Nigeria.
[3]National Hospital Abuja, Nigeria.

***Corresponding Author:**
Jude Ogechukwu Okoye
Tel: +2347031119994,
Email: jog.okoye@unizik.edu.ng

Received: 3/3/2024; accepted for publication 21/4/2024

Abstract

Background: Although many drugs exist for Benign Prostate Hyperplasia (BPH) today, it remains the most common prostatic disease affecting older men. Herbal medicine has become more common as an affordable alternative therapy for curing and preventing tissue-related pathologies in hopes of overcoming the side effects of existing synthetic pharmaceutical products and surgical procedures. This study evaluated the effects of single and combined ethanolic extract of *Curcuma longa* and *Moringa oleifera* on testosterone-induced BPH, urothelial, and testicular toxicity in albino rats.

Method: In this experimental study BHP was induced in albino rats and treated with *C. longa*, *M. oleifera*, *C. longa*/*M. oleifera*, and Tamsulosin hydrochloride. Serum prostate-specific antigen (PSA) and tissue p63 expression were estimated by the ELISA and Avidin-Biotin Complex (immunohistochemistry) methods. Differences in PSA levels among the Groups were assessed using ANOVA and significance set at $p< 0.05$.

Result: Biochemical findings showed significantly increased serum PSA levels in BPH induced group (0.59 ± 0.07 nmol/L) without treatment compared with BPH-induced group treated with a single extract of *C. longa*, *M. oleifera*, combined extracts of *C. longa* and *M. oleifera* and standard drug (0.12 ± 0.06 nmol/L, 0.16 ± 0.04 nmol/L, 0.11 ± 0.02 nmol/L, and 0.20 ± 0.04 nmol/L, respectively) at $p< 0.05$. Histology revealed giant cell formation in the BPH-induced group without treatment. Combined administration of a mixture of *C. longa* and *M. oleifera*

showed an observable reduction of the p63 protein expression in urothelial cells when compared with the single use of either of the plants.
Conclusion: This study revealed that combining both *C. longa* root and *M. oleifera* seed extracts has curative potential on experimentally induced BPH. It suggests that standardized herbal medicine could be used as an alternative to orthodox medicine, especially in low-resource settings.

Keywords*:* *Curcuma longa, Moringa oleifera*, Benign Prostate Hyperplasia, p53 protein, testes

Introduction

Benign Prostate Hyperplasia (BPH) is the proliferation of stromal and glandular cells, characterized by an excessive increase in the number of smooth muscles within the prostate region.[1] Protein 63 (p63) is found in the nuclei of basal cells of normal urogenital cells. It is a key regulator in cell proliferation and cell differentiation in stratified squamous epithelium.[2] It is overexpressed in proliferating basal cells of epithelial layers in the epidermis, cervix, urothelium, nasal epithelium and prostate.[3] More than 300 active components have been identified in Turmeric (*Curcuma longa*) but the component of interest is curcumin (diferuloylmethane).[4] It is obtained from its roots, and it is commonly used to treat wounds, inflammation, and some tumours due to its antioxidant content.[5] *Curcuma longa* reduces testicular damage caused by an increase in glutathione (GSH), testosterone levels, and glucose-6-phosphate dehydrogenase activity and a decrease in malondialdehyde (MDA) levels.[6] The main limitation of using Curcumin as an antiproliferative agent is its low oral bioavailability due to its extensive first-pass metabolism and its poor aqueous solubility.[7] An adjuvant known as piperine (the most active component of *Piper nigrum*, black pepper), which is a known inhibitor of hepatic and intestinal glucuronidation has been advocated. Simultaneous administration of piperine (20 mg/kg) increased the serum concentration of curcumin with a bioavailability of 154% in rats and 2000% in humans with concomitant decreases in its clearance.[8,9] *Moringa oleifera* is cultivated worldwide for its nutritional properties.[10] Its leaves and seeds are traditionally used to treat various ailments, including abdominal tumours, scurvy, paralysis, prostate and bladder problems, sores, and skin infections due to its antiproliferative, antioxidant and anti-inflammatory.[11,12] Studies have shown that *M. Oleifera* and *C. longa* complement each other in terms of the amount of inherent phytochemical compounds and minerals: Anthocyanin, Carotenoids, Cardiac glycoside, Saponins, Alkaloids, Steroids, Flavonoid, Terpenoids, Tannins and Anthraquinone, and Zinc, Iron, Phosphorus, Magnesium, Potassium, Calcium and Nitrogen.[13-15] In recent times, combination therapy has been practised to improve disease conditions which were mildly affected by single therapy. One such therapy included a combination of genistein and curcumin which prevented cellular proliferation caused by a single or mixture of pesticides. This study mainly evaluated the combined effect of *C. longa*, and *M. oleifera* on artificially induced BPH and Testicular toxicity.

Materials and Methods

Animal handling

A total of 54 male albino rats weighing 150 to 180 g were used for this study. The animals were purchased and bred in the

Animal Faculty of Babcock University. Rats were housed in wooden cages, under standardized housing conditions 12 hours light and 12 hours darkness. The rats were fed twice daily with clean tap water and chow *ad libitum*. They were allowed to acclimatize for 14 days before the experiment. The actual experiment lasted for 2 weeks. The experimental procedures adopted were following the Babcock University Health Research Ethics Committee (BUHREC530/18), Babcock University, Ilishan-Remo, Ogun State and the US National Institute of Health Guidelines for Care and Use of Laboratory Animals in Biomedical Research.

Plant collection, preparation, and extraction

Tumeric *Curcuma longa* roots, *M. Oleifera* seeds and black pepper *Piper nigrum* fruit were obtained from the Ilishan community market and identified at the Department of Agriculture, Babcock University. The plants were dried at 40^{o}C, and pulverized into powder, mixed with 70% ethanol (in a ratio of 1:8) and with intermittent agitation for 72 hours. The solids were then filtered out using a filter and a vacuum pump for accelerated filtration. The solution was concentrated with a rotary evaporator at 60^{0}c. After most of the ethanol had been removed, the solution was poured into a glass petri dish and placed in an oven overnight at 40^{0}c to remove moisture further.

Study design

Following acclimatization, 54 male Albino rats were randomly divided into 9 groups and treated as follows: Group 1 (control): 1 ml/kg of distilled water for 14 days, Group 2: 5 mg/kg of Testosterone Propionate (TESTOST^{TM}; Laborate Pharmaceuticals Ltd., India) for 7 days and sacrifice after another 7 days (Li et al., 2018), Group 3: 25 mg/kg of *C. longa*+ 25 mg/kg of piperine (Pp)for 7 days, Group 4: 5 mg/kg of Testosterone Propionate for 7 days and 25mg/kg of *C. longa* + 25 mg/kg of piperine for another 7 days, Group 5: 50 mg/kg of *M. oleifera* for 7 days, Group 6: 5 mg/kg of Testosterone Propionate for 7 days and 50 mg/kg of *M. oleifera* for 7 days, Group 7: 25 mg/kg of *C. longa* + 25 mg/kg of piperine+25 mg/kg of *M. oleifera* for 7 days, Group 8: 5 mg/kg of Testosterone Propionate for 7 days and 25 mg/kg of *C. longa* + 25 mg/kg of piperine+25 mg/kg of *M. oleifera* for 7 days. Group 9: 5 mg/kg Testosterone Propionate for 7 days and 50 mg/kg of Tamsulosin hydrochloride (standard BPH drug from Contiflo XL^{TM}; Ranxaby Ltd., United Kingdom). Administration of Testosterone Propionate was done subcutaneously. Distilled water, *C. longa* root extract, *M. oleifera* seed extract, piperine, and Tamsulosin hydrochloride were administered orally with the aid of an oral cannula. The study lasted for 28 days. Of note, piperine was used to improve the bioavailability of *C. longa*.

Sample Collection and Investigation

After two weeks, the animals were subjected to an overnight fast and anaesthetized by drop method. Blood samples were collected through the jugular vein and discharged in plain tubes. Blood samples were allowed to clot, and the sera were separated. Animals were then sacrificed by cervical dislocation and organs (prostate and testes) were harvested, weighed, fixed in neutral buffered formalin, and processed. Photomicrographs of the tissues were taken for documentation. The serum level of Prostate Specific Antigen (PSA) was determined by ELISA method using a commercial kit (Accu-Bind^{TM}; Monobind Inc., USA). The Avidin-Biotin Immuno-peroxidase method, as described by Okoye et al.,[16] was used to demonstrate the presence of p63 gene expression in basal

cells of the prostate by using kits: 1) Anti-p63 [BSH-3006-S, clone BSR6, Nordic Biosite AB, Sweden/Ref.: TL-925-QPB160718], 2) DAB Quanto substrate [Ref.: TA-125-QHSx160718; Thermo-Fisher scientific], 3) Citrate buffer [Ref.: AP-9003-125; Thermo-Fisher scientific], 4) Phosphate buffered saline buffer with Tweenzo [Ref.: ab64028], DAB chromogen [ab80436, GR171088-3; Abcam Nigeria], and 5. Hydrogen peroxide Solution B [Batch № 2356; Samstella Ind. Nig Ltd, Nigeria].

Statistical Analysis

The differences between the experimental groups were assessed using ANOVA (SPSS, version 20). The prostate and testis indices were calculated by dividing the organ weight by the body weight multiplied by 100.

Results

The administration of testosterone propionate induced benign prostate hyperplasia with a concomitant increase in the prostate index ($F= 0.92$, $p= 0.51$) and testes index ($F= 0.99$, $p= 0.46$), though the increases were not statistically significant ($p>0.05$) when compared to other groups. Results showed significantly elevated levels of PSA among animals in group 2 (BPH untreated group) compared with other treatment groups and control ($F= 2.19$, $p= 0.05$) and an insignificant decrease in body weight of animals in group 2 ($F= 1.86$, $p= 0.10$) (table 1). The single administration of *M. oleifera* or *C. longa* extract resulted in reduced levels of PSA after the experiment while administration of a combination of both extracts resulted in a minimal increase in PSA level when compared with the control group. Results also showed an observable correlation between biochemical, histopathological, and immunological findings (Figure 1).

Table 1: Comparison of PSA levels and Body weights before and after the experiment (mean ± SD)

Group (G)		Parameters					
	Description of Groups	**PSA before experiment (PB; ng/ml)**	**PSA after experiment (PA; ng/ml)**	**Mean Diff. in PSA levels (PA-PB; ng/ml)**	**Body weight (WB; g)**	**PI (%)**	**TI (%)**
G1	Control (D/w)	0.01 ± 0.01	0.06 ± 0.03[b]	0.05 ± 0.02	194.2 ± 15.99	0.24	1.61
G2	TP 7 days, sacrifice after 7 days	0.10 ± 0.03	0.59 ± 0.07	0.49 ± 0.71	173.20 ± 14.45	0.37	2.06
G3	*C. longa* (CL) for 7 days	0.06 ± 0.02	0.02 ± 0.02[a]	-0.04 ± 0.00	203.60 ± 11.89	0.25	1.74
G4	TP 7 days, Cl for 7 days	0.05 ± 0.03	0.12 ± 0.06[a]	0.07 ± 0.03	201.60 ± 16.79	0.27	1.68
G5	*M. oleifera* (Mo) for 7 days.	0.15 ± 0.05	0.02 ± 0.01[b]	-0.13 ± -0.04	192.80 ± 15.93	0.29	1.56
G6	TP 7 days, Mo for 7 days	0.04 ± 0.02	0.16 ± 0.04[a]	0.12 ± 0.03	208.33 ± 27.70	0.22	1.46
G7	Cl/Mo for 7 days	0.06 ± 0.04	0.08 ± 0.03[b]	0.02 ± 0.00	203.75 ± 11.93	0.23	1.67
G8	TP 7 days, Cl/Mo for 7 days	0.06 ± 0.02	0.11 ± 0.02[b]	0.05 ± 0.00	208.20 ± 20.87	0.24	1.58
G9	TP for 7 days, STD for 7 days	0.04 ± 0.02	0.20 ± 0.04[a]	0.16 ± 0.02	201.60 ± 17.05	0.28	2.02

Statistically significant at P< 0.05= a, P<0.01= b, ANOVA, N= 54, n= 6

KEY: G; Group**, Diff.; Difference, CL;** ***C. longa*****, Mo**; *M. oleifera,* **TP**; Testosterone Propionate, SD; Standard deviation, **STD**; Standard Drug (Tamsulosin hydrochloride), **N;** total number of animals, **n**; number of animals per group, **D/w**; Distilled water, TI; Testis index, PI; Prostate Index

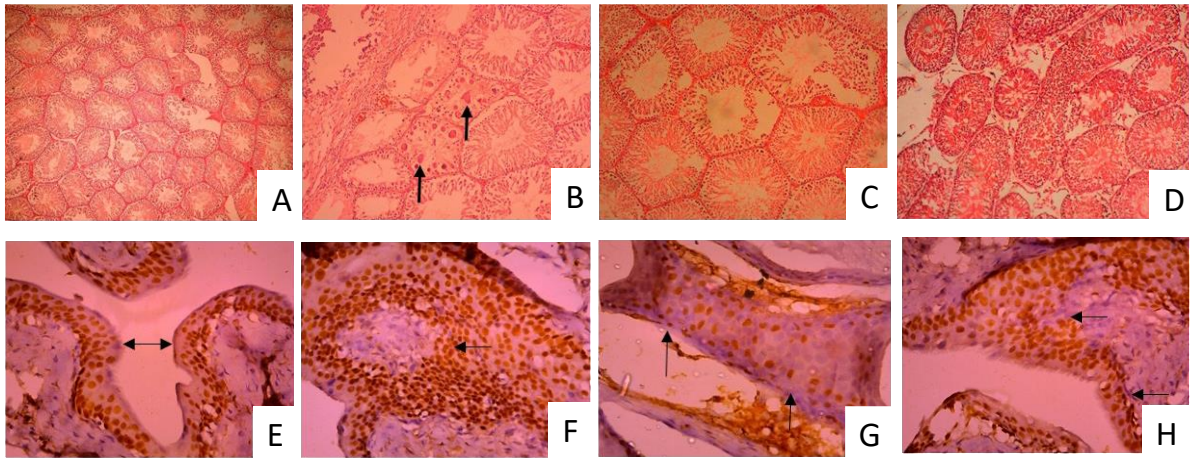

Figure 1: Histology of Testis and urethra

Figure 1A: Photomicrograph of a testis section from the control group (G1) showing normal maturation and well-differentiated seminiferous tubules (Stained by H&E technique, X100 magnification). Figure 1B (G2; BPH induced group without treatment) shows moderate depletion but severely distorted seminiferous tubules, mild haemorrhage, and giant cell formation (black arrows). Figure 1C (BPH-induced group treated with *C. longa/M. oleifera* treated group; G8): shows moderately depleted and distorted seminiferous tubules. Figure 1D (G9; BPH induced group, treated with standard drug): Photomicrograph shows mildly distorted seminiferous tubules (Stained by H&E technique, X200 magnification). Figure 1E: Photomicrograph of a urethra section negative for stromal expression of p63 protein. Figure 1F: Photomicrograph of urothelial cells (G2) with evidence of high p63 protein expression. Figure 1G shows mild p63 protein in urothelial cells following treatment with *C. longa/M. oleifera.* Figure 1H: moderate expression of p63 protein in urothelial cells after treatment with the standard drug (Stained by immunohistochemical technique, X200 magnification).

Discussion

The prevalence of BPH rises markedly with age with a histological prevalence of 8%, 50% and 80% in the 4th, 6th and 9th decades of life, respectively.[17] A survey carried out among men above 40 years in Nigeria (South-West) showed a BPH prevalence of 23.7%.[18] Although many drugs exist to cure BPH today, it remain the most common prostatic disease affecting older men.[19] Natural drugs have become more common as an alternative method for curing and preventing BPH in hopes of overcoming the side effects of existing synthetic pharmaceutical products and surgery procedures. Since the administration of *C. longa* root and *M. oleifera* seed extracts resulted in a considerable reduction in PSA levels in the experimental animals, it was hypothesized that a combination of both may further reduce serum PSA levels. This present study assessed the effect of the combined administration of *C. longa* and *M. oleifera* on testosterone-induced benign prostate hyperplasia and testicular injury in male albino rats. It estimated serum PSA level, prostate index and testes index as parameters for confirming BPH induction.[20-22] The observed high PSA level, prostate index, testes index, and epithelial hyperplasia were obvious indications that BPH was successfully achieved following the administration of testosterone propionate for seven days. The increased testes index in the untreated group when compared with the control group is an indication of BPH because of the relationship existing between the testes and prostate; testicular androgens contribute to the growth of the prostate gland.[23,24]

The serum PSA level of all treatment groups decreased differently after 7 days of administering the plant extracts and standard drug. The extracts, therefore, seem to have inhibited cell proliferation and induced apoptosis in the animals following treatment. Biochemical findings in this study show that turmeric and *M. oleifera* when administered singly reduced serum PSA levels. Histological results were also in concordance with the biochemical findings since there was decreased hyperplasia and papillary formation.[25] The potential of *M. oleifera* seed extract to reduce PSA levels in the experimental animals is in concordance with an earlier study which showed that ethanolic leaf extract of *M. oleifera* forestalled artificially induced BPH by improving the antioxidant effect and inhibiting inflammatory mediators.[26] Despite the reduced serum PSA levels observed among animals that received single therapy of *C. longa* or *M. oleifera*, the combination of both extracts did not show any significant decrease in the assayed PSA levels. However, histological findings in the prostate and testes suggest otherwise in that there was a marked reduction in observable papilloma and testicular injury. The immunohistochemical result suggests that combined therapy of *C. longa* root and *M. oleifera* seed extracts reduced the expression of the p63 gene more than the single therapy of either of the two compared with the standard drug.[27-30]

Conclusion

This study revealed that combining both *C. longa* root and *M. oleifera* seed ethanolic extracts are less toxic, reduces PSA levels and downregulates the p63 gene. Thus, *C. longa* and *M. oleifera* should be incorporated into the meals of BPH patients and high-risk individuals to reduce the progression of prostate disease and patients' mortality.

Competing interest

No competing interests exist in this study.

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