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Research Article

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Evaluation of Tumour-Inhibiting Effect of Ethanolic Extract of *Cyperus Esculentus* on *Agrobacterium*-Induced Tumour on Potato Disc

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Abstract

The study investigated the antineoplastic effect of ethanolic extract from *Cyperus esculentus* tuber on *Agrobacterium*-induced tumour in potato discs. The research was based on the Reactive oxygen species scavenging potentials of methanolic, ethanolic, aqueous, n-hexane and ethyl acetate extracts of *Cyperus esculentus* (0.2-1.0 mg/ml) *in vitro* using DPPH radical, hydrogen peroxide and ferric ion reducing assay. Phytochemical analysis (Total phenol and flavonoids) revealed the methanolic extract produced highest amount of total phenol (0.245), followed by ethanolic extract (0.208) while aqueous yielded 0.032 and ethyl acetate was 0.041. The flavonoids content with absorbance value between 0.397 and 0.075 with methanolic extract producing the highest yield. Extracts of *C. esculentus* seeds produced concentration dependent scavenging effect on both DPPH and H_2O_2 (0.8 mg/ml) aqueous extract, (1.0 mg/ml) n-hexane and ethanol, while 0.4 mg/ml) methanol and (0.2 mg/ml) ethyl acetate produced 85.42%, 54.44%, 52.65%, 33.79% and 27.25% scavenging effect on DPPH radical respectively and 1.0 mg/ml aqueous extract produced highest scavenging effect at 82.5%, followed by 0.2 mg/ml ethanolic extract at 46.14% and 0.2 mg/ml methanolic extract produced scavenging effect on H_2O_2 at 42.85%. The reducing power of aqueous extract was most significant. Anti-tumour assay showed that the ethanolic extract have tumour-inhibitory effect on *Agrobacterium*-induced tumour on potato discs in a concentration dependent pattern with maximum tumour inhibition at 0.5 mg/ml ethanolic extract concentration against *Agrobacterium tumefaciens* Strain LBA 4404. However, the extract did not show any lethal activity against *Agrobacterium tumefaciens* strains, hence, the assay is acceptable for the evaluation of the antineoplastic activity of various crude extract eliciting ROS scavenging and reduction potentials.

Keywords

Cyperus esculentus; *Agrobacterium tumefaciens;* potato-disc antitumor; Antioxidant

Introduction

Biological activities such as growth and metabolic processes in living organisms as well as exogenous factor generate by-products such as singlet oxygen (O_2), superoxide anion (O_2^-) and hydroxyl (OH) radical and hydrogen peroxide (H_2O_2) which are collectively referred to as Reactive oxygen species (ROS). They are highly reactive and can cause serious chemical damage to DNA, proteins, and unsaturated lipids. ROS intermediates have been implicated in a number of pathogenic processes, including reperfusion injury, cancer, inflammatory disease, and aging. These lead to a shift in homeostasis and when left unattended to oxidative damage. In response to the generation of ROS, nature has several protective mechanisms that serve to minimize the toxic potential of these compounds. They could be enzymatic e.g. superoxide dismutase (SOD), catalase or non-enzymatic e.g. glutathione. Under disease conditions or other pathological processes including aging as well as exposure to exogenous sources of free radical, these protective mechanisms are disrupted, making the need for external antioxidant supplements vital to effectively combat oxidative damage.

Antioxidants are agents/chemicals that inhibit the destructive effect of oxidative free radicals by interacting with and neutralizing them, thus preventing them from causing damage. Also referred to as "free radical scavengers," they bind with free radical forming complexes that can be easily excreted from the body. They also possess ability to repair damages caused by these free radical and stop further oxidation reactions by being oxidized themselves. Antioxidants are capable of stabilizing, or deactivating, free radicals before they attack cells. They are essential for maintaining optimal cellular and systemic health and well-being [1].

To protect cells and organs of the body against ROS, humans have evolved a highly sophisticated and complex antioxidant protection system. When generated within the body, they are called endogenous antioxidants. However, the body relies on external (exogenous) sources, primarily the diet, to obtain the rest of the antioxidants it needs. These exogenous antioxidants are commonly called dietary antioxidants, which are mainly of plant origin. Dietary sources of antioxidants includes fruits such as carrots, citrus, berries; vegetables such as spinach, cucumber, rich in vitamin C and E. Metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions that are capable of catalyzing oxidative reactions. Numerous other antioxidant phytonutrients are present in a wide variety of plant foods.

Tiger nut (*Cyperus esculentus L.*) has been studied for its medicinal values. Oil and tubers of *C. esculentus* are slightly sweet and nutty flavour. It is been used as a foodstuff, particularly in Africa, where it is an important food crop with certain tribes. The nut is a tough erect fibrous-rooted perennial plant, 1 to 3 ft high, reproducing by seeds and by many deep, slender rhizomes, which form weak runners above the ground, and small tubers or nutlets at the tips of underground stems. The tubers are about the size of peanuts and are grown in Nigeria. Other names of the plant are earth almond, yellow nut, Hausas call it Aya, Yorubas imumu, and Igbos calls it ofio [2].

C. esculentus like most plants contain desired biological activities, which in most cases are due to a mixture of bioactive plant

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components. Therefore, screening crude extracts for biological activity is an essential step towards the development of new drug. For their investigation, it is important to have the suitable biological assays as a follow up for *in vitro* assays. Initial screening for antitumor potential by using *Agrobacterium tumefaciens* (crown gall tumor formation on potato discs) could be used as a fairly rapid, inexpensive and reliable pre-screen for antitumor agent. The potato disc bioassay also serves as an efficient alternative to extensive animal testing in the search for new anticancer drugs.

Potato disc assay was shown to be useful for checking known and novel antitumor molecules' properties. This bioassay is based on *Agrobacterium tumefaciens* infection on potato disc [3].The validity for the use of such assay is that the tumorogenic mechanism initiated in plant tissues by *A. tumefaciens* is in many ways similar to that of animals [4]. In fact, Kempf et al., [5] showed that *Bartonella henselae*, a bacterium causing tumor in human shares a similar pathogenicity strategy, with plant pathogens *A. tumefaciens*. Several studies are finding numerous and significant areas of similarity among the mechanisms used by bacterial pathogens of plants and humans. These similarities include the use of common toxins, secretion system, adhesion mechanism, invasion and regulation [6].

Materials and Methods

Plant materials

Dried seeds of *Cyperus esculentus* were obtained from lafenwa market, Lafenwa, Abeokuta, Ogun state. Fresh russet potatoes (*Solanum tuberosium L.*) were obtained from Oja-Oba market, Osogbo, Osun state.

Chemicals and solvents

Methanol, ethanol and ethyl acetate were purchased from Tianjin kermel Chemical Reagent Co., Ltd, n-hexane was purchased from Scharlau S.L., Gato Perez, 33-P. I. Mas d'En Cisa, 2,2-diphenyl-1,1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Inc., St. Loius, MO, 3,5-dimethyoxy-4-hydroxy-acetophenone (acetosyringone) was purchased from fluka, and dimethyl sulphoxide (DMSO) was purchased from SMM chemicals (pty) limited. Bacto agar was purchased from Difco laboratories, Detroit Michigan, USA, Nutrient broth powder and yeast extract.

Extraction

The dried seeds of *C. esculentus* were collected and surface area was increased to course powder by grinding in a blender. 50 g of dried powdered seeds were separately extracted with water, ethanol, methanol, ethyl acetate and n-hexane. The various solvent preparation of *C. esculentus* sample was prepared by pouring 500 ml of the respective solvents upon 50 g of powdered sample and agitated for 12 hrs at 150 rpm to obtain maximum extraction before filtration. The extracts were subsequently concentrated by using the sohxlet apparatus, and further concentration was done in a water bath at 100°c.

DPPH scavenging assay

The method described by Yen and Duh [7]. Aliquots of 2.5 ml of sample solution (ethanolic, methanolic, aqueous, n-hexane and ethyl acetate extracts of *C. esculentus*) were measured into test tubes protected from the light. Freshly prepared ethanolic solution of DPPH (1 ml, 0.28 mM) was added to the mixture. The solution was mixed properly and left undisturbed to react in the dark for 30 min at room temperature. The absorbance (Abs_{sample}) was read at 518 nm using a spectrophotometer. The blank was prepared by mixing 1 ml of ethanol with 2.5 ml sample solution. The negative control was obtained by mixing 1 ml of DPPH solution and 2.5 ml of ethanol. The absorbance of these solutions was read (Abs_{blank}, $Abs_{control}$). The spectrophotometer was zeroed with ethanol (99.5%).

$$DPPH\ scavenging\ effect\ (\%)=\frac{Abs_{control}\left(Abs_{sample}-Abs_{blank}\right)}{Abs_{control}}\times 100$$

Hydrogen Peroxide scavenging assay

Hydrogen peroxide scavenging activity of methanolic, ethanolic and aqueous extracts of *C. esculentus* seeds was evaluated according to the method of Ruch et al. (1989). Samples (methanolic, ethanolic and aqueous extracts of *C. esculentus* seeds) (3.4 ml) dissolved in phosphate buffered saline (pH 7.4) were mixed with 0.6 ml of H_2O_2. The absorbance was read at 230 nm after 10 minutes.

$$Percentage\ hydrogen\ peroxide\ scavenging\ effect=\frac{Abs_{control}\left(Abs_{sample}-Abs_{extract}\right)}{Abs_{control}}\times 100$$

Reducing power

The reducing power of the methanolic, ethanolic, aqueous, ethyl acetate and n-hexane extracts of *C. esculentus* seeds was determined using the method described by Oyaizu [8]. Sample solution (0.5 ml) at different doses were mixed with phosphate buffer (0.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (0.5 ml, 1%). The mixture was then incubated at 50°C for 20 minutes. A portion of trichloroacetic acid (0.5 ml, 10%) was added to the mixture, which was then centrifuged at 402 x *g* for 10 minutes. The upper layer of the solution (0.5 ml) was transferred to a fresh tube, and mixed with distilled water (0.5 ml) and $FeCl_3$ (0.1 ml, 0.1%) then incubated at room temperature for 10 minutes, and then the absorbance was read at 700 nm in a spectrophotometer, with higher absorbance indicating greater reducing power.

Determination of total phenols

Total phenol content in the samples was determined using Folin-Ciocalteu's method as described by Olajire and Azeez [9]. 0.5 ml (1 mg/ml) of extract was added to a mixture of 10 ml distilled water and 2.5 ml of 10% Folin-Coicalteu's reagent. The mixture was incubated at room temperature for 5 minutes. 2 ml of 2% $NaCO_3$ was added. The absorbance of the resulting solution was taken at 780 nm. Quercetin was done in three concentrations and the absorbance was taken in triplicate.

Determination of flavonoids

The $AlCl_3$ method of Jagadish et al. (2009) was used for the determination of the total flavonoids content of the extract samples. 1.5 ml of extract was added to 1.5 ml of 2% methanolic $AlCl_3$ solution. The mixture was vigorously shaken on orbital shaker for 5 minutes at 200 rpm. The absorbance was read at 367 nm after 10 minutes of incubation. Quercetin was used as standard for the calibration curve. The content of the flavonoid compound were expressed as mg quercetin/g of extract. The assay was repeated thrice.

Potato disc tumour induction assay

The method described by Coker et al. [10] was used to determine the anti-tumour activity of ethanolic extract of *C. esculentus* seeds. *A. tumeficeiens* was grown on Nutrient-Yeast media for 48 hours at

28°C. Russet potatoes (*Solanum tuberosium L.*) were disinfected by scrubbing in water bowl to remove soil dirt, peeled and immersed in 10% bleach for 5 minutes. Potatoes were cut into circular discs of 5 mm×8 mm, immersed in 2% bleach for 30 minutes, rinsed in sterile distilled water by soaking in water and agitating for 15 minutes and repeated thrice, then blot dry with sterile paper towels. Four potato discs were placed in each plate of three well cultured plates containing 28 g/L bacto-agar in nutrient yeast broth. Each plate represented positive control, negative control and experimental plates. Positive control plate was overlaid with sterile distilled water only. Negative control plate was divided into two groups of two discs each, a group overlaid with well growing *A. tumeficeiens* and the other *A. tumeficeiens* + inducer- acetosyringone. Experimental plate was divided into four groups of one disc each, each group overlaid with 0.001, 0.01, 0.0001 and 0.5 mg/L extract in solvent respectively. Plates were incubated for 11 days. Readily prepared Lugol's reagent is overlaid on potato discs. Presence of dark-blue/black colouration indicates carbohydrate molecules.

Results and Discussion

Compelling evidence indicates that increased consumption of dietary antioxidants or fruits and vegetables with antioxidant properties may contribute to the improvement in quality of life by delaying onset and reducing the risk of degenerative diseases associated with aging. Phenolics are the most wide spread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. It was reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers [11]. Therefore, in the present study, total phenolic content present in extract was estimated by using Folin-Ciocalteu's method as described by Olajire and Azeez [9]. The content was observed to vary with the solvent of extraction; methanolic extract (0.245%), ethanolic extract (0.208%) while aqueous yielded 0.032% and ethyl acetate was 0.041%. The flavonoids content was between 0.397% and 0.075% with methanolic extract producing the highest yield (Figure 1). However, free radical scavenging analysis with all assay trends away from this as methanolic extract showed least scavenging ability.

Scavenging effect on DPPH radical is one of the models for testing for antiradical properties of chemical compounds including plant extract. Thus, the DPPH radical scavenging activity produced by the methanolic, ethanolic, aqueous, n-hexane and ethyl acetate extracts of *C. esculentus* seeds is an indication that it can terminate lipid peroxidation by donating hydrogen (Figure 2). The ROS ($^{\cdot}OH^{-}$, $O_2^{\cdot-}$ and H_2O_2) scavenging activities methanolic, ethanolic, aqueous, n-hexane and ethyl acetate extracts of *C. esculentus* seeds in this study shows its possible potentials to complement the ROS detoxifying enzymes *in vivo,* if effectively absorbed. It could also help terminate lipid peroxidation that could arise from $O_2^{\cdot-}$ and the stronger ROS ($O^{\cdot}$, $^{\cdot}OH$ and H_2O_2) [7]. Also, the capability of methanolic, ethanolic, aqueous and ethyl acetate extracts of *C. esculentus* seeds to significantly reduce $K_3Fe(CN)_6$, shows its effectiveness to halt the oxidation of cellular macromolecules by oxidizing molecules that could arise from the metabolism of either drugs or toxins (Figure 3). Reactive oxygen species (ROS) was implicated in pathogenic processes such as carcinogenesis and tumourigenesis. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The decomposition of H_2O_2 by *Cyperus esculentus* tuber may at least partly result from its antioxidant and free radical scavenging activity (Figure 4). The formation of the ferrozine-Fe^{2+} complex is interrupted in the extract of *Cyperus esculentus* tuber, indicating its chelating activity, Chelating agents that forms bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion [12]. This study shows that extract of *Cyperus esculentus* tuber has a marked capacity for iron binding, suggesting the presence of polyphenols that has potent iron chelating capacity.

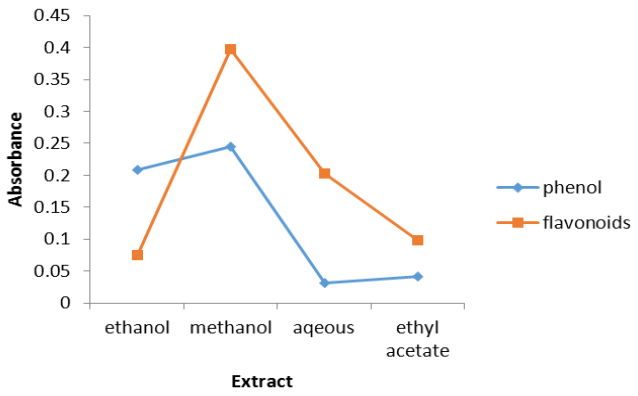

Figure 1: Total phenol and flavonoids content of *C. esculentus* ethanolic, methanolic, aqueous and ethyl acetate extracts.

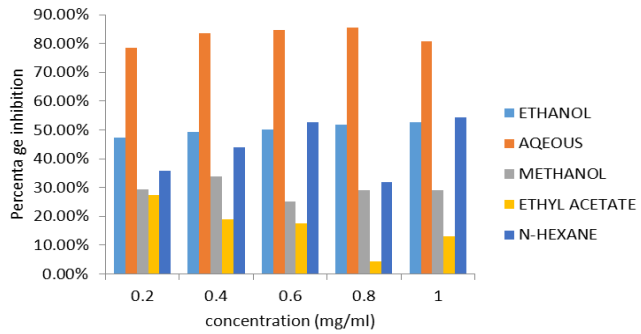

Figure 2: DPPH scavenging effect on *C. esculentus* methanolic, ethanolic, aqueous, n-hexane and ethyl acetate extracts.

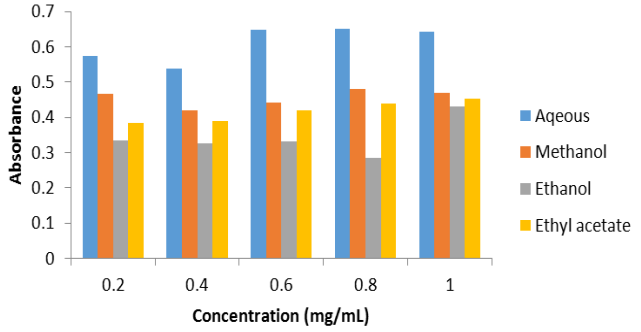

Figure 3: Reducing power of *C. esculentus* methanolic, ethanolic and aqueous extracts.

Based on the convincing evidence observed from the antioxidant assay as well as reports from previous works, the antineoplastic effect of ethanolic extract from *Cyperus esculentus* tuber on *Agrobacterium*-induced tumor in potato discs was carried out to verify the biological activities of the bioactive component of extract of *Cyperus esculentus* tuber. This assay can be routinely employed as a comparatively rapid, inexpensive, safe and statistically reliable pre-screen for 3PS (*in vivo* murine leukemia) antitumor activity. From the study carried out, it was observed that extracts of *C. esculentus* show antitumor potential in terms of the phenotypic effects. Potato disc assay was carried out using *Agrobacterium tumefaciens* for tumor induction. Various *Agrobacterium* strains can be used for this purpose, however in the present study, *A. tumefaciens* strains LBA 4404 was used against four different concentrations (0.5 mg/ml, 0.01 mg/ml, 0.001 mg/ml and 0.0001 mg/ml) of the extract, and there was a significant difference in tumor inhibition by the four concentrations of extract. Antitumor assay showed that the ethanolic extract have tumor-inhibitory effect on *Agrobacterium*-induced tumor on potato discs in a concentration dependent manner with maximum tumor inhibition at 0.5 mg/ml ethanolic extract concentration against *Agrobacterium tumefaciens* Strain LBA 4404 (Plate 1). However, the extract did not show any lethal activity against *Agrobacterium tumefaciens* strains, hence, the assay is acceptable for the evaluation of the antineoplastic activity of various crude extract eliciting ROS scavenging and reduction potentials.

The tumorogenic mechanism initiated in plant tissues by *A. tumefaciens* is in many ways similar to that of animals [4]. *A. tumefaciens* utilizes a mechanism similar to the one used by *Bartonella henselae*, a tumour-causing bacterium in human [5]. Ability of ethanolic extract of *C. esculentus* to scavenge free radical *in vitro* is an indication of possible tumour-inhibition property. Ethanolic extracts of *C. esculentus* produced concentration dependent inhibition of *A. tumefaciens*-induced tumour in potato discs. Higher concentration of ethanolic extract of *C. esculentus* showed higher tumour inhibition. Mechanism-base assays detect potential anticancer agents that interfere with neoplastic growth, or focus on target receptors that were discovered as the mechanism of drug action. Crown Gall is a neoplastic disease of plants induced by specific strains of *A. tumefaciens*. Galsky et al. first demonstrated that inhibition of Crown Gall tumor distribution on potato discs correlated with compounds and plant extracts known to be active in the 3PS leukemic mouse tumor assay. The antitumor effect is independent of antibiosis. Thus, as a pre-screen, antibiosis is not a problem. The potato Disc Tumor Assay is a simple, inexpensive and fast screen for antitumor agents. It was used to identify the antitumor activity of ellagic acid and an extract of *Moliva volkensii* fruit. The study reported herein affirms the use of this assay as a first general screen in the search for new antitumor agents, irrespective of their mode of inhibition.

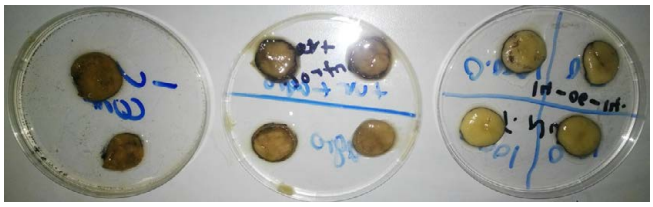

Plate 1: Lugol's Reagent Test on Tumour Induced Potato Discs.

Lugol's Reagent test: From left to right; negative control (potato discs + sterile distiled water only), positive control (potato discs + *A. tumefaciens* + acetosyringone) and experimental group (potato discs + ethanolic extract of *C. esculentus* seeds + *A. tumefaciens*).

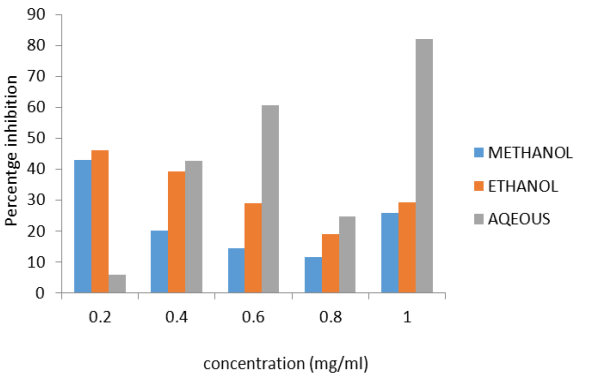

Figure 4: Scavenging Effect of *C. esculentus* Methanolic, Ethanolic and Aqueous Extracts on Hydrogen Peroxide.

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