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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Short Communication

Allelopathic effects of *Cassia tora* and *Cassia uniflora* on *Parthenium hysterophorous* L.

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Laboratory studies on the seed powder and water extract of seeds of *Cassia tora* and *Cassia uniflora* on the germination of *Parthenium* seeds was studied. Seed powders of both weeds were much inhibitory on seed germination of *Parthenium* than the water extract. Seed powder of *Cassia tora* showed maximum germination inhibition of 98% and water extract of 67%, whereas seed powder of *C. uniflora* showed germination inhibition of 95% and water extract of 61%. The results showed that the *C. tora* seed powder and water extract of its seeds showed much inhibitory effect than *C. uniflora* on *Parthenium*.

Key words: *Cassia tora*, *Cassia uniflora*, seed extract; *Parthenium hysterophorus*;

INTRODUCTION

In plant protection, allelochemicals play a vital role. In floral community there is much influence at biodiversity and composition level. Allelochemicals vary at various stages of plant and also in different parts of plants, in particular stages of development. Out of many competitors, widely found Cassia species are widely distributed herbaceous weeds common in India wastelands.

Cassia tora and *Cassia uniflora* cover a large area of wastelands. Cassia species can be used for the biological control of *Parthenium hysterophorus*. Plant parts of *C. tora* have allelochemicals which inhibit seed germination of *Parthenium* (Kumar and Bhan, 1997; Senthil et al., 2004; Singh and Thapar, 2002).

Biochemical substances are mostly secondary metabolites by plant in primary metabolism intermediates pathway called allelochemicals. There are several ways in which allelopathic plants can release their protective chemicals which include volatilization, leaching, exudation, etc. (Akhtar et.al, 2001).

Of these various ways, weed-weed interaction is an important phenomenon which was observed in case of *Cassia uniflora* and *Cassia tora* dominates on *Parthenium* and *Achyranthus aspera* dominates on *C. uniflora*.

P. hysterophorous L. commonly called congress grass is a vigorous colonist in poor and overgrazed pastures and readily occupies bare areas where stock movement is frequent. It does not invade most well managed crops and pastures, but may be a problem even when present at low levels. *Parthenium* weed inhibits the growth and germination of other plants by allelopathy.

Parthenium weed is known to cause allergic reactions in people (Mahadevappa and Patil, 1999). Symptoms are itching on exposed skin and development of a dermatitis which may spread over the whole body. It also causes asthma. The sesquiterpene lactone parthenin is the major allergen produced. *C. tora* and *C. uniflora* were tested for antigermination activity by allopathic effect on *P. hysterophorous* L.

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Table 1. Germination of *Parthenium hysterophorous* seeds exposed to *Cassia tora* seeds powder.

Test No.	Seed germination in control (%)	Seed germination in seed powder test (%)	Seed inhibition in seed powder (%)
1	90	00	100
2	90	4	96

Table 2. *Cassia tora* seed powder water extract.

S/N	Seed germination in control (%)	Seed germination in seed powder test (%)	Seed inhibition in seed powder (%)
1	90	30	70
2	90	35	65

Table 3. Germination of *Parthenium* seeds exposed to *Cassia uniflora* seed powder.

S/N	Seed germination in control (%)	Seed germination in seed powder test (%)	Seed inhibition in seed powder (%)
1	90	3	97
2	90	7	93

MATERIALS AND METHODS

Collection of plant

The seeds of *C. tora* and *C. uniflora* were collected from pods from University of Pune campus. The seeds were obtained from pods, dried and then powdered for carrying out further experiments.

Parthenium plant was collected from University of Pune campus seeds which were obtained from these plants. The seeds were dried and powdered in a pestle and mortar.

Preparation of water extract

Water extract of seeds of *C. tora* and *C. uniflora* was prepared by distilling the seed powder with water in a Soxhlet distillation apparatus.

Apparatus used

Sterile petri plates, germination paper, Soxhlet distillation apparatus was used for the extraction of seed powder.

Experimental

Preparation of seeds powder

The seeds of *C. tora* and *C. uniflora* were dried in the sun and powdered.

Preparation of water extract

Fifty grams of seed powder of each plant was taken in a round bottom flask containing 500 g of water and an extraction was carried out in a Soxhlet distillation apparatus for 8 h. The extract was then filtered and used for experimentation.

Germination test

Healthy and equal sized seeds of *P. hysterophorous* were taken in a beaker. The seeds were soaked in hot water (50 ml) up to 5 min. Out of these, 100 seeds were taken for germination test. Treated seeds are sown in petri plate containing germination paper and water extract was taken and petriplate was then kept in germination chamber at temperature of about (22 to30°C).

Similarly in control, seeds without water extract were taken with distilled water for 7 days. Both petri plates were kept in germination chamber for 7 days. Two laboratory germination tests were conducted at 25°C.

RESULTS AND DISCUSSION

The results of the experiment are tabulated as shown in Tables 1 to 4. The data on the seed powder and seed powder water extract of *C. tora* and *C. uniflora* on the germination of *Parthenium* seeds is as shown in Tables 1 to 4.

Seed powders of both weeds were much inhibitory on seed germination of *Parthenium* than the water extract. Seed powder of *C. tora* showed maximum germination inhibition of 98% and water extract of 67%, whereas seed powder of *C. uniflora* showed germination inhibition of 95% and water extract of 61%. The results showed that the *C. tora* seed powder and water extract of the seeds showed much inhibitory effect than *C. uniflora*.

It can also be concluded that allelochemicals extracted from *C. tora* and *C. uniflora* have the potential to inhibit the germination of *Parthenium* seeds. *C. tora* and *C. uniflora* showed allelopathic effect on *Parthenium*. Both plants are found in abundance in nature. By using water extract or simply seed powder, inhibition of *Parthenium*

Table 4. Germination of *Parthenium hysterophorous* seeds exposed to *Cassia uniflora* seed powder water extract.

S/N	Seed germination in control (%)	Seed germination in seed powder test (%)	Seed inhibition in seed powder (%)
1	90	42	58
2	90	36	64

takes place which is confirmed by this experiment.

In water extract of the seeds and seed powder, some allelochemical substances are present which affect germination of *Parthenium*. In future, Cassia species can act as herbicide to other weed such as *Parthenium*. For agricultural practices, both are more beneficial, because they are abundant in nature and have high inhibition property to *Parthenium*.

The allelochemicals present in extract affected germination and seedling growth (Narwal et al., 1992). The allelochemicals present in extract might have altered some of the physiological processes responsible for the plant growth.

REFERENCES

Akhtar N, Javaid A, Bajwa R (2001). Herbicial activity of aqueous extracts of *Cirsium arvense* and *Ageratum conyzoized* against weeds of wheat. Pak. J. Biol. Sci. 4:1364-1367.

Kumar S, Bhan VM (1997). 'Natural *Parthenium* replacement by *Cassia tora* at Jabalpur and adjoining areas of Madhya Pradesh in India'. In: Proceedings of the First International Conference on *Parthenium*.

Mahadevappa M, Patil VC (1999). Management University of Agricultural Sciences, Dharwad, Karnataka, India. 2:41- 43.

Narwal SS, Pahuja SS, Gupta K (1992). 'Allelopathic effects of stubble extract of pearl millet on seed germination and seedling growth of fodder crops'. In: Proceedings of the First National Symposium on Allelopathy in Agro ecosystems, Tauro, P. and S. S. Narwal (Eds.). Indian Society of Allelopathy, Haryana Agricultural University, Hisar, India. p. 21.

Senthil A, Chinnusamy C, Shanmugasundaram R, Kandasamy OS (2004). 'Identification of competitive or allelopathic plant species for the management of *Parthenium hysterophorus*'. In: Abstracts of the IV international conference allelopathy in sustainable terrestrial and aquatic ecosystems, Narwal, S. S. (Ed.). International Allelopathy Foundation, Haryana Agricultural University, Hisar, India. p. 24.

Singh NB, Thapar R (2002). 'Allelopathic effects of *Cassia* on germination, growth and metabolism of *Parthenium*'. Iran. J. Plant Physiol. 3:4.

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Full Length Research Paper

Anti-inflammatory effects and mechanisms of usnic acid, a compound firstly isolated from lichen *Parmelia saxatilis*

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Usnic acid (UA) was isolated for the first time from *Parmelia saxatilis* and was confirmed by physical and spectral evidence. Its anti-inflammatory effect and mechanism were explored on lipopolysaccharide (LPS)-stimulated RAW264.7 cell line. The effects of UA on pro-inflammatory cytokines including tumor necrosis factor-alfa (TNF-*α*), interleukin-6 (IL-6) and interleukin-1 beta (IL-1*β*), pro-inflammatory mediators such as nitric oxide (NO), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were studied by sandwich enzyme-linked immunosorbent assay (ELISA), real-time polymerase chain reaction (PCR) and Western blot analyses. Similarly, the effect of UA on anti-inflammatory cytokine interleukin-10 (IL-10) and anti-inflammatory mediator heme oxygenase-1 (HO-1) were also studied following the same methods. Furthermore, nuclear factor-κB (NF-κB) was assayed by immunocytochemistry. The results showed that UA has anti-inflammatory effect by down-regulatinng iNOS, COX-2, IL-1*β*, IL-6 and TNF-*α*, COX-2 gene expression through the suppression of NF-κB activation and increasing anti-inflammatory cytokine IL-10 and anti-inflammatory mediator HO-1 production.

Key words: *Parmelia saxatilis*, usnic acid, anti-inflammation.

INTRODUCTION

Lichens have been used for medicinal purpose throughout the ages. As lichens are symbiotic associations between fungus and alga (photobiotic), a number of lichens were screened for antibacterial activity in the 1940s and 1950s following the discovery of penicillin from a fungus (Vartia, 1973) and several compounds were found to be active against mycobacterium species and Gram-positive bacteria. Due to significant antibacterial activity, some extracts of lichens or isolated compounds were used as antibiotics clinically. *Parmelia saxatilis* belonging to the genus *Parmelia* was widely used in folk medicine for hundreds of years to treat fever and lumbar pain. However, phytochemical (Ingolfsdotir et al., 1998) and pharmacological reports of *P. saxatilis* are rare. During the course of searching for anti-inflammatory substances, we found the extract of *P. saxatilis* having potent anti-inflammatory activity. Following bioassay guided investigations on bioactive substances, Usnic acid (UA) was first isolated from *P. saxatilis*.

UA is well-known antibiotic, endowed with several biological and physiological activities including antiviral, antibiotic and anti-inflammatory (Ingolfsdtir, 2002). However, the anti-inflammatory activity and mechanism

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of UA have rarely been researched further (Ingolfsdtir, 2002; Li et al., 2007). Hence studying the anti-inflammatory mechanism of UA was also undertaken as an objective of the present study.

Inflammation is a multiple process, mediated by activated inflammatory factors or immune cells. During its course, macrophages and monocytes usually play crucial roles in eliciting response cascade in the acute phase of inflammation (Baumann and Gauldie, 1994). After being stimulated, they produce a number of chemokines and enzymes, such as TNF-*α*, IL-1*β* (Bertolini et al., 2001; Mongan et al., 2000), IL-6 (Stadnyk et al., 1997) and IL-10 (Hofman, 2004; Inui et al., 2002), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Kröncke et al., 1998; Kim et al., 2003), and anti-inflammatory mediator (HO-1) (Ogborne et al., 2004), which is macrophage or monocyte-related cytokine, essential for the inflammatory response to pathogenic germs or toxicants. Activation of pro-inflammatory as well as anti-inflammatory cytokines and mediators is the key procedure of inflammatory reaction which subsequently lead to inflammatory impairment and restoration (Naldini and Carraro, 2005). It is well known that nitric oxide (NO) is synthesized by iNOS and involves in diverse physiological processes. An excess NO production is largely thought of causing a variety of inflammatory diseases (Clancy et al., 1998). NF-κB is a nuclear transcription factor regulating the expression of various genes including IL-1*β*, IL-6, TNF-*α* and iNOS, which play critical roles in apoptosis, tumor genesis and various autoimmune diseases and inflammation (Lawrence et al., 2001; Riehemann et al., 1999; Renard and Raes, 1999). Thus, suppression of these factors may be an effective therapeutic strategy for preventing inflammatory reaction and diseases (Makarov, 2000). The variation of NF-κB activity could be used for reflecting the anti-inflammatory effect of the inhibitors.

In this study, LPS-stimulated RAW264.7 cell line was used as an inflammation cellular model to explore the anti-inflammatory effect of UA and anti-inflammatory mechanism (Kim et al., 2003).

MATERIALS AND METHODS

Plant

The lichen *P. saxatilis* was purchased from Hubei province herb Co. Ltd. China and identified by Professor Keli Chen (Hubei College of Traditional Chinese Medicine).

Extraction and isolation

The whole air-dried plants (1.0 kg) were extracted at room temperature with 95% ethanol for 2 days. After removal of ethanol in vacuum, the residue was extracted with petroleum ether, then the petroleum ether fraction was condensed in vacuum and the yellow crystal was precipitated. The filtered crystalline precipitate was washed by e4thyl acetate for several times and then recrystallizated with double-distilled water and ethyl acetate. The structure of the crystal was determined using spectroscopic techniques including electron ionization mass spectrometry (EI-MS), ^{1}H and ^{13}C NMR, heteronuclear multiple bond correlation (HMBC) spectrometry and X-ray diffraction analysis (Figure 1).

Cell lines, chemicals and biochemicals

Murine macrophage cell line RAW 264.7 was obtained from the China Center for typical culture collection (Wuhan, China). Roswell Park Memorial Institute (RPMI) 1640 was purchased from Gibico (Grand Island, USA). Lipopolysaccharide (LPS) (*Escherichia coli* O111:B4) and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma Co. (St Louis, USA). Affinity-purified goat anti-mouse HO-1 antibody was obtained from R&D Systems (Minneapolis, MN). Affinity-purified goat anti-mouse COX-2 antibody, rabbit anti-mouse NF-κB antibody was purchased from Santa Cruz Biotechnology (CA, USA). *β*-actin was purchased from MBI Ferments Company (prestained protein maker: # SM0441, MW was117.0 ,90.0, 49.0, 35.0, 26.0 and 19.0 KDa, MD, USA). Mouse TNF-*α*, L-1*β* and IL-6 ELISA kits were purchased from Quantikine, R&D Systems (detection limit, 1 pg/ml, Minneapolis, USA). Griess reagent nitric oxide (NO) assay kit was purchased from Jingmei Biotech Co., Ltd (Shenzhen, China). Mouse IL-10 ELISA kit was obtained from Bender Medsystem (detection limit, 1 pg/ml, Vienna, Austria).

Preparation of cells

The cells were maintained in RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin and 10% fetal bovine serum. Cells were cultured with 5% CO_2 in humidified air at 37°C. The crystal of UA was accurately weighed and diluted by 1640 medium to concentrations of 10, 5 and 1 µg/ml, which were then used for treatment in LPS-stimulated RAW 264.7 cell line. Before treatment by LPS, the cells were inoculated into 6, 24 or 96 micro-well plates. 24 h later, the cells were observed to be adhering to the bottom of wells; the cell supernatants were disposed from the wells, and then 10 ng/ml LPS with prepared different doses of UA was added to the wells: 10, 5 and 1 µg/ml. There were four kinds of control groups, including positive control group where cells treated with DEXAMETHASONE (DM, 0.5 µg/ml), negative control group, cells treated with *Astragalus* polysaccharides (APS, 100 µg/ml), blank control group, cells only stimulated by LPS (10 ng/ml) and normal control group where cells incubated by 1640 medium.

Determination of anti-inflammatory activity

MTT assay for the measurement of cell proliferation

Cytotoxic effect of UA was evaluated by conventional MTT assay (Mosmann, 1983). Meanwhile, cell cytopathic effect (CPE) test of the cells was studied using the microplate. Cytotoxicity determinations were conducted by seeding RAW 264.7 cells into microplates at 4×10^3 cells per well. After an overnight incubation, compound in MEM containing 10% fetal bovine serum (FBS) was added (contents of the compound were 10, 50 and 100 µg/ml, respectively).

Cells were allowed to grow for an additional 24 h. At 4 h prior to culture termination, 20 µl of the MTT solution (5 mg/ml in phosphate-buffered saline, pH 7.4) was added and the cells were continuously cultured until termination. 150 µl dimethyl sulfoxide (DMSO) was added into each well for the solubilization. The optical density (OD) at 490 nm was measured by a Spectramax 250 microplate reader.

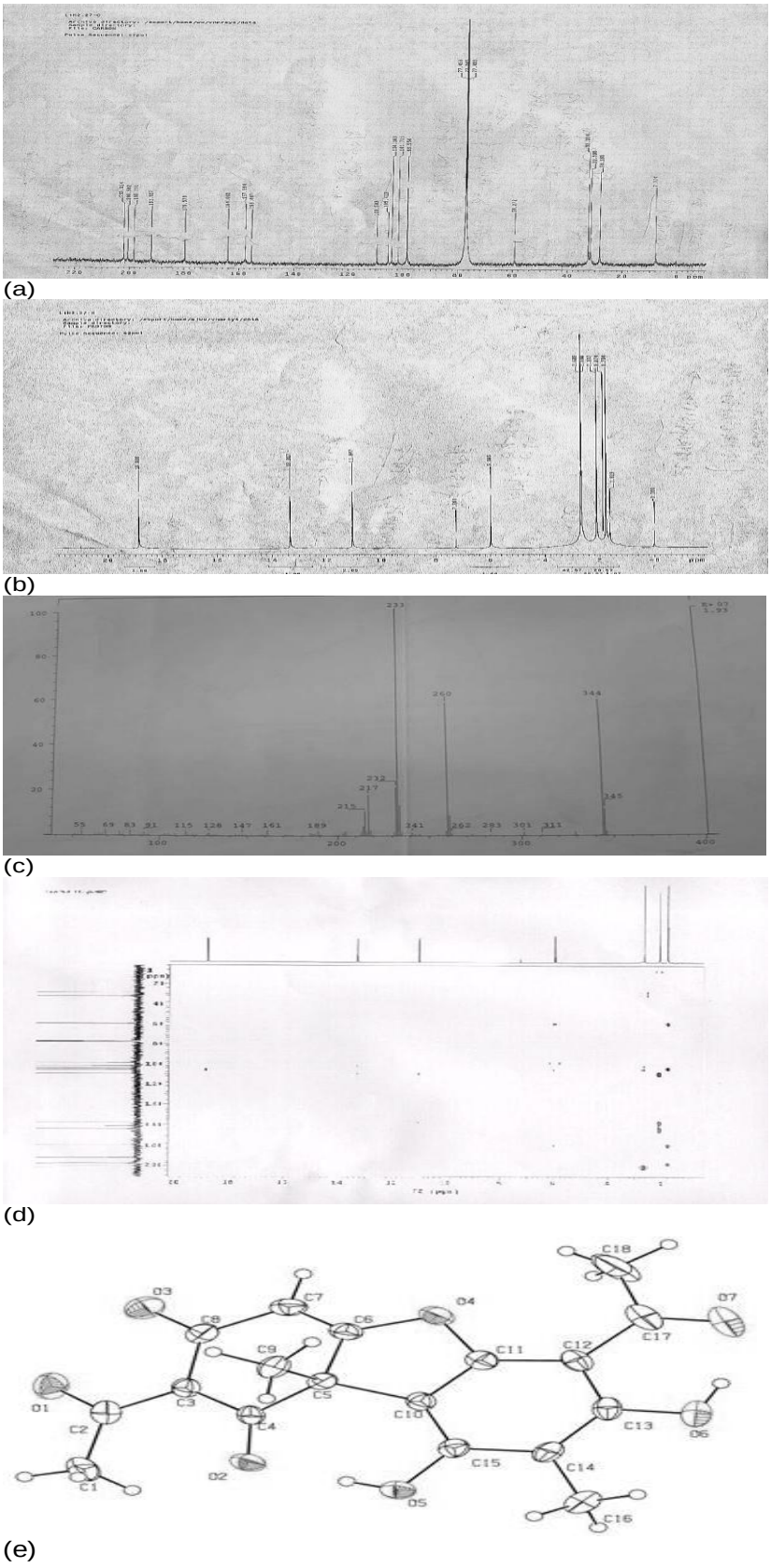


Figure 1. Structure identification of usnic acid (UA). (a) ^{1}H NMR spectrum of UA, (b) ^{13}C NMR Spectrum of UA, (c) MS spectrum of UA, (d) HMBC of UA, (e) X-ray of UA.

Detection of cytokines TNF-α，IL-1β , IL-6 and IL-10 production

After stimulation and treatment on RAW 264.7 cells by LPS with different concentrations of UA for 24 h, cell supernatants of the cell culture were collected and assayed for TNF-*α*, IL-1*β*, IL-6 and IL-10 by the sandwich ELISA method with ELISA kits according to the instructions provided by the manufacturer.

Determination of NO production

Levels of NO were determined by the Griess reaction (Green et al., 1982). The samples were assayed in triplicate by a nitrite detection kit according to instructions provided by the manufacturer, and a standard curve was generated in each experiment using $NaNO_2$.

Quantitative real-time PCR for detecting mRNA of TNF-α, COX-2, iNOS and HO-1

TRIzol Reagent (Gibco BRL) was added according to manufacturer's protocol and stored at −80°C before use. The total RNA for detection of pro-inflammatory factors TNF-*α*, iNOS and COX-2 were extracted at 4 h after the cells were stimulated with LPS and treated by UA; 18 h after the cells stimulation and treatment was for extracting the RNA of HO-1. Quantitative real-time polymerase chain reaction (PCR) was performed in a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) with the FastStart DNA Master SYBR Green I kit (also from Roche), and the results were analyzed with LDCA software supplied with the machine (Livak and Schmittgen, 2000) [23]. The primers used were detailed in Table 1 (Zhao et al., 2007).

Western blot analysis of COX-2 and HO-1

RAW 264.7 cells were incubated with or without LPS in the presence or absence of UA for 24 h. The cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended in PBS containing 0.1 mM phenylmethylsulfonyl fluoride. They were laid by three cycles of freezing and thawing in liquid nitrogen. The cytokine fractions were obtained from each supernatant after 12,000 g centrifugation at 4°C for 20 min. Samples (30 μg protein) were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBST (0.1%) for 1 h and then incubated with polyclonal antibody for goat HO-1 (1:6000 dilutions) or polyclonal antibody for COX-2 (1:6000 dilutions) in TBST containing 1% nonfat milk for 1 h. The membranes were then hybridized with secondary antibody conjugated with horseradish peroxidase (anti-rabbit and anti-mouse IgG-HRP, 1:2000 dilutions, Santa Cruz Biotechnology, USA) for 1 h and washed five times with TBST. The membranes were immediately incubated with ECL detection kits (Pierce Biotechnology Co., Ltd, USA) for 2 min and later exposed to X-ray film.

Immunocytochemical assay on NF-κB

Covers slips were placed in the wells to allow the cells crawl on them. After treatment with LPS and UA, the cells were washed, fixed and blocked. The first antibody (anti-mouse NF-κB IgG) (diluted to 1:100) was added then. 12 h later, the cells were mixed with the horseradish peroxidase-labelled second antibody (biotinylated anti-IgG) (diluted to 1:75) at room temperature for 30 min. Then the colorization with diaminobenzidine, counterstaining with hematoxylin, ethanol dehydration (orderly by 75, 95 and 100%), dimethyl benzene clearance and mounting observation were performed in sequence. Each sample was randomly chosen at 5 visual fields in microscope and positive cells were counted. NF-κB was detected 24 h after treatment with UA.

Statistical analysis

Student's *t*-test was used in determining the statistical significance of differences between the values for the various experimental and control groups. Data were expressed as means ± standard deviation (SD) and p-values of 0.05 or less were considered to be statistically significance.

RESULTS AND DISCUSSION

From petroleum ether fraction of ethanol extract of *P. saxatilis*, a crytalline compound was isolated which was confirmed as usnic acid by comparing its physical and spectral data with authentic literature (Kumar et al., 1996). It is the first time that usnic acid was found occuring in *P. saxatilis*.

In vitro cytotoxicity of UA from *P. saxatilis*

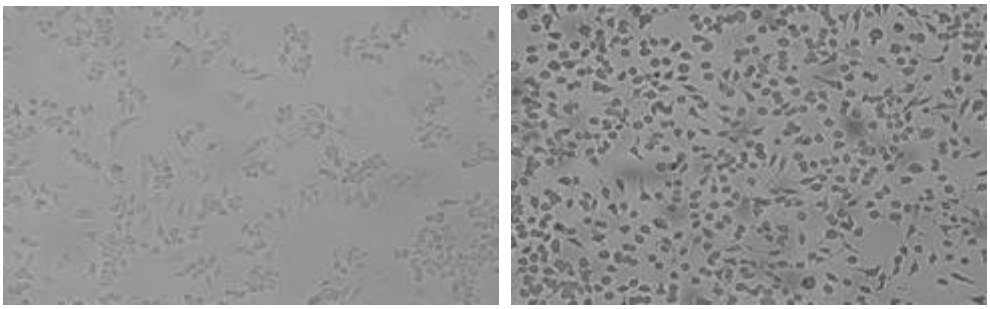
Pre-treatment of unstimulated RAW264.7 cells with UA of no more than 10 μg/ml for 24 h did not significantly affect cell viability. The cytopathic effect (CPE) test also gave the same result (the content of the compound was no more than 10 μg/ml) (Figure 2). The result suggested that UA have no cytotoxicity to RAW264.7 cell line.

Effects of UA on the pro-inflammatory cytokines and mediators produced by LPS stimulation

After the cells were stimulated by LPS and treated with UA for various times, it was found that UA significantly decreased the secretion of TNF-*α*, IL-1*β*, IL-6 and NO compared to single LPS stimulation ($p < 0.01$ or $p < 0.05$) (Figure 3a to d). Furthermore, the higher the dose of UA, the greater the influence on antagonizing pro-inflammatory cytokines, such results showed a dose-dependent relation of UA on anti-inflammatory effect. Similarily, UA strikingly decreased the levels of COX-2 protein, TNF-*α* mRNA and COX-2 mRNA compared to blank control ($p < 0.01$ or $p < 0.05$) (Figure 3e to g), which suggested that UA might control pro-inflammatory factors production not only at levels of proteins but also at transcriptional and translational level. Furthermore, the effect of UA on iNOS mRNA (Figure 3h) was coincidental with that on NO (Figure 3d).

Effects of UA on anti-inflammatory cytokines and mediators produced by LPS stimulation

The IL-10 levels of cells treated with UA were similar to that of DM treatment, which showed UA and DM had a



(a) (b)

Figure 2. Results of CPE of usnic acid (UA). (a) The cell before treatment by UA, (b) The cell after treatment by UA (1 × 40 times in microscope).

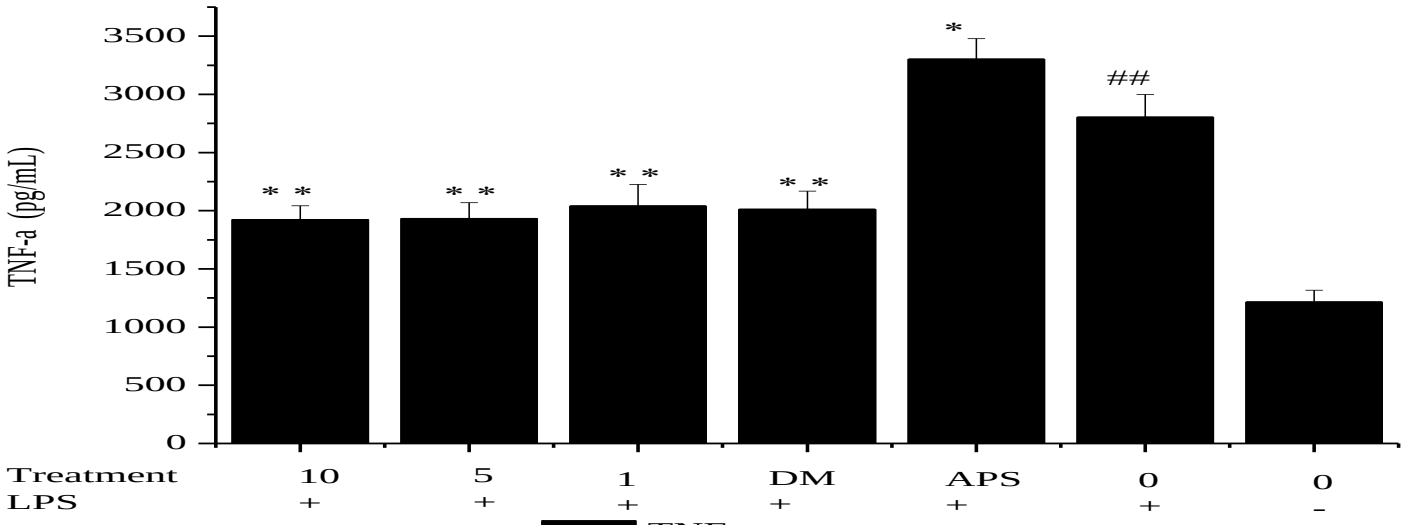


Figure 3. Effects of UA on TNF-α production. RAW264.7 cells were treated with LPS (10 ng/mL) in the presence of various concentrations of UA. TNF-α were measured in the cell culture media by ELISA.
Data shown were the mean ± SD (n= 3). *p<0.05; **p<0.01 versus LPS alone, $^{\#}p$<0.05 or $^{\#\#}p$<0.01 compared to normal cell.

similar inhibitory effect on anti-inflammatory cytokine (Figure 4a). The levels of HO-1 mRNA and protein in UA treatment and DM and APS treatment cells were significantly higher than those in single LPS stimulation and normal group cells ($p < 0.05$) (Figure 4b and c). Furthermore, the higher the dose of UA, the greater the effect on expression of HO-1 mRNA and protein, which suggested that UA might promote regression of inflammation and control anti-inflammatory mediators production at levels of proteins and by transcriptional and translational in a dose-dependent manner.

Effect of UA on NF-κB of usnic acid

NF-κB activation was significantly blocked by UA (Figure 5), which suggested that the suppression of IL-1β, IL-6, TNF-α, iNOS and COX-2 gene expression by UA might be due to the attenuation of NF-κB activation. Though UA is commonly occuring in Lichens, this is the first time that the compound is isolated from *P. saxatilis*. We firstly found that UA could inhibit TNF-α, IL-1β and IL-6 in LPS-stimulated RAW 264.7 cells; these pro-inflammatory cytokines and mediators possess a multitude of biological activities linked to the immunopathology of acute or chronic inflammatory diseases (Bertolini et al., 2001; Plutzky, 2001). These results suggested that the observed changes of proinflammatory cytokines productions might be associated with anti-inflammatory effect of UA. Furthermore, we studied the effect of UA on anti-inflammatory cytokines and mediators, such as IL-10 and HO-1 (Zhao et al., 2007), and found that it could increase the production of IL-10 and HO-1 protein (and gene levels, which illustrates the dual effects of UA on antagonizing

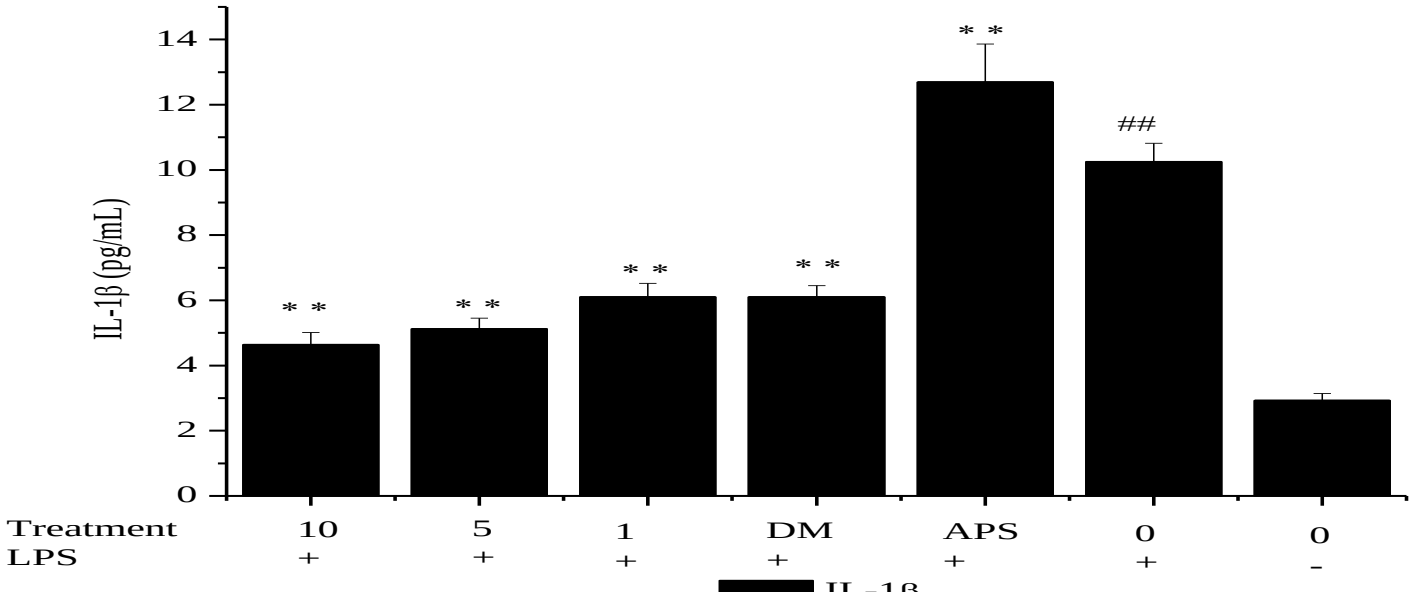


Figure 4. Effects of UA on IL-1β production. RAW 264.7 cells were treated with LPS (10 ng/ml) in the presence of various concentrations of UA. IL-1β was measured in the cell culture media by sandwich ELISA.
Data shown were the mean ± SD (*n* = 3). *p < 0.05; **p < 0.01 versus LPS alone, $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ compared to normal cell.

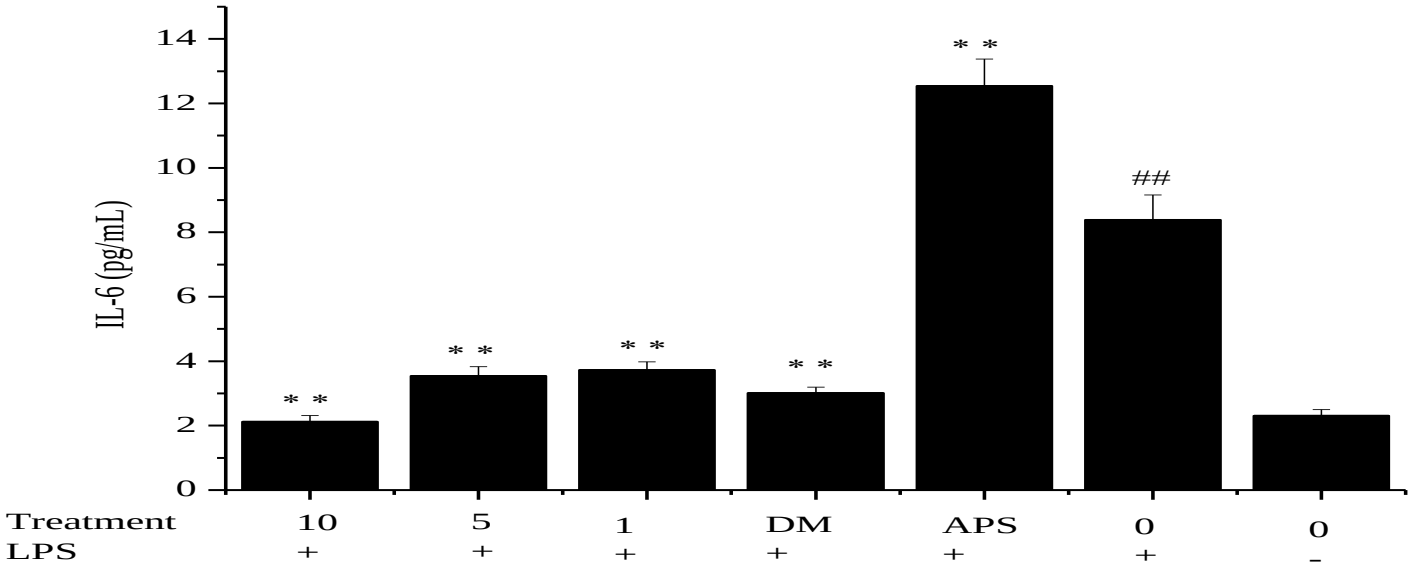


Figure 5. Effects of UA on IL-6 production.RAW 264.7 cells were treated with LPS (10 ng/ml) in the presence of various concentrations of UA. IL-6 levels were measured in the cell culture media by ELISA.
Data shown were the mean ± SD (n = 3). *p < 0.05; **p < 0.01 versus LPS alone, $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ compared to normal cell.

antagonizing pro-inflammatory and augmenting anti-inflammatory mediators.

The results also showed that UA significantly inhibited the gene expression of iNOS and COX-2, which resulted in the suppression of NO production in LPS-stimulated RAW264.7 cells. Meanwhile, NF-κB is a nuclear transcription factor that regulates the expression of various genes, including IL-1*β*, TNF-*α*, iNOS and COX-2 (Figures 6 to 14) during inflammation process. We also investigated whether UA could inhibit NF-κB production or not. The

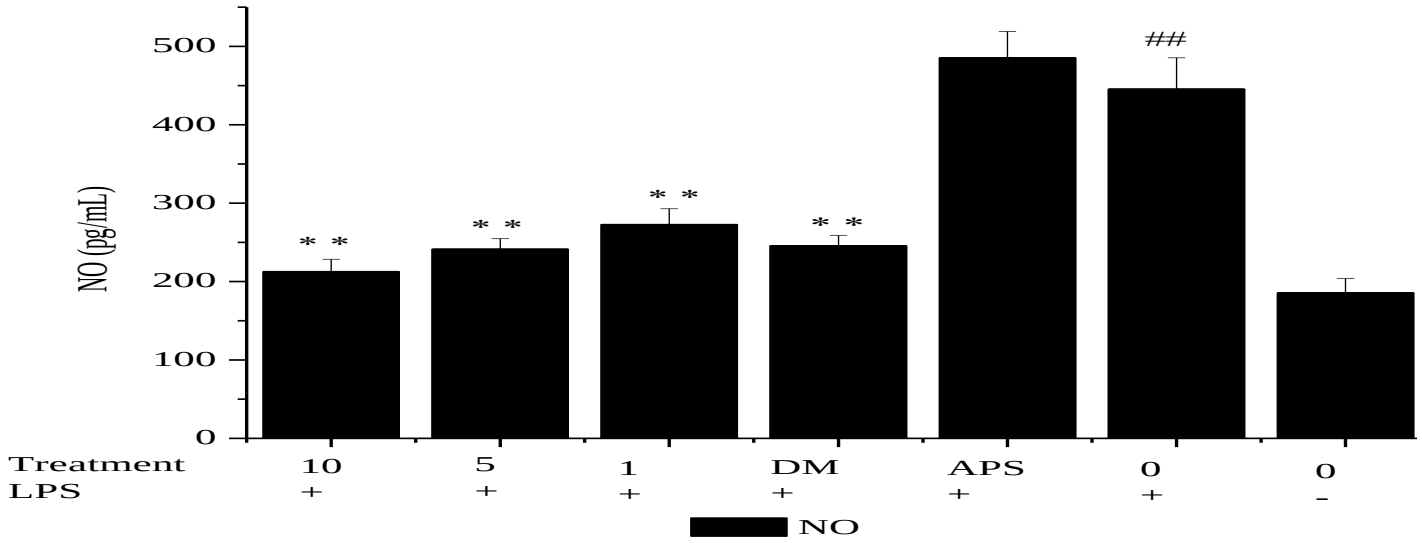


Figure 6. Effects of UA on on NO production. RAW264.7 cells were treated with LPS (10 ng/ml) in the presence of various concentrations of UA. NO levels were measured in the cell culture media by ELISA.
Data shown were the mean ±SD (n = 3). *p < 0.05; **p < 0.01 versus LPS alone, $^{\#}p$ < 0.05 or $^{\#\#}p$ < 0.01 compared to normal cell.

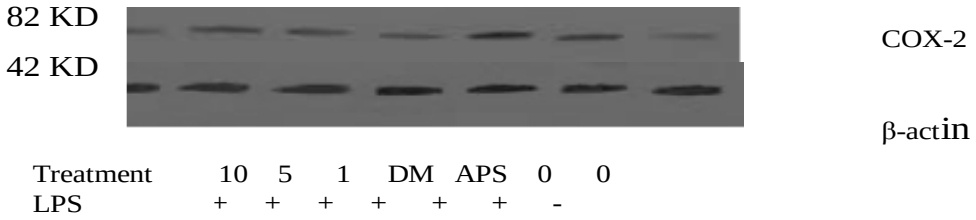


Figure 7. Effects of UA on COX-2 protein production. RAW264.7 cells were treated with LPS (10 ng/ml) in the presence of various concentrations of UA. COX-2 protein levels were measured in the cell culture media by Western blot analysis.

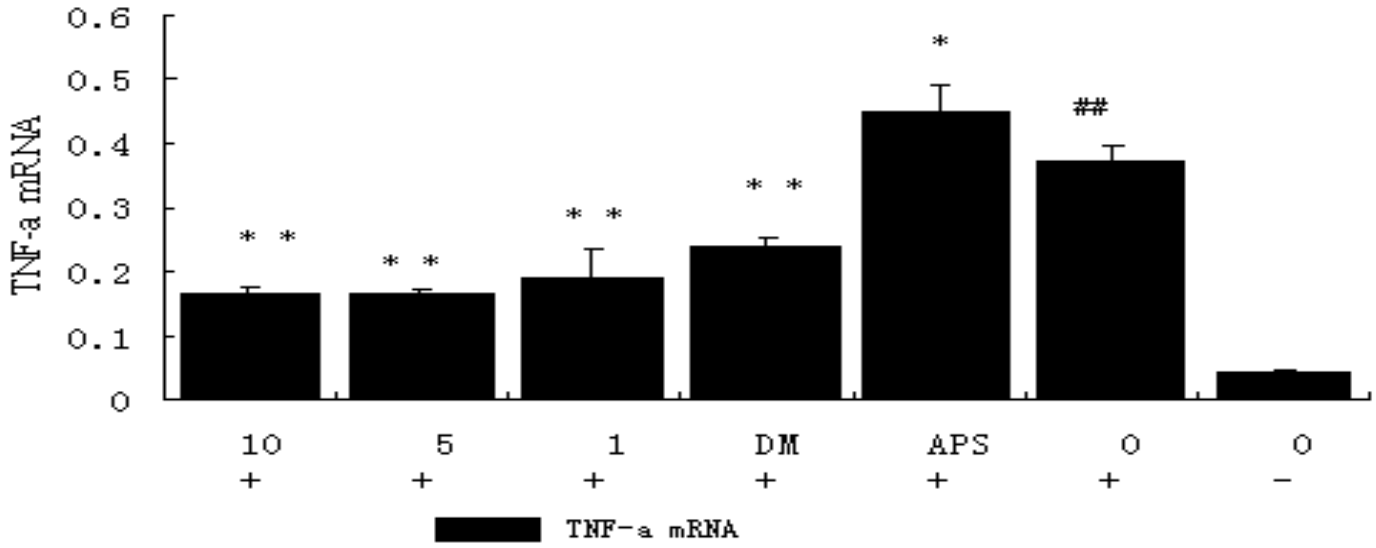


Figure 8. Effects of UA on TNF-α mRNA production. RAW264.7 cells were treated with LPS (10 ng/ml) in the presence of various concentrations of UA. TNF-α levels were measured by real time PCR.
Data shown were the mean ± SD (n= 3). *p < 0.05; **p < 0.01 versus LPS alone, $^{\#}p$ < 0.05 or $^{\#\#}p$ < 0.01 compared to normal cell.

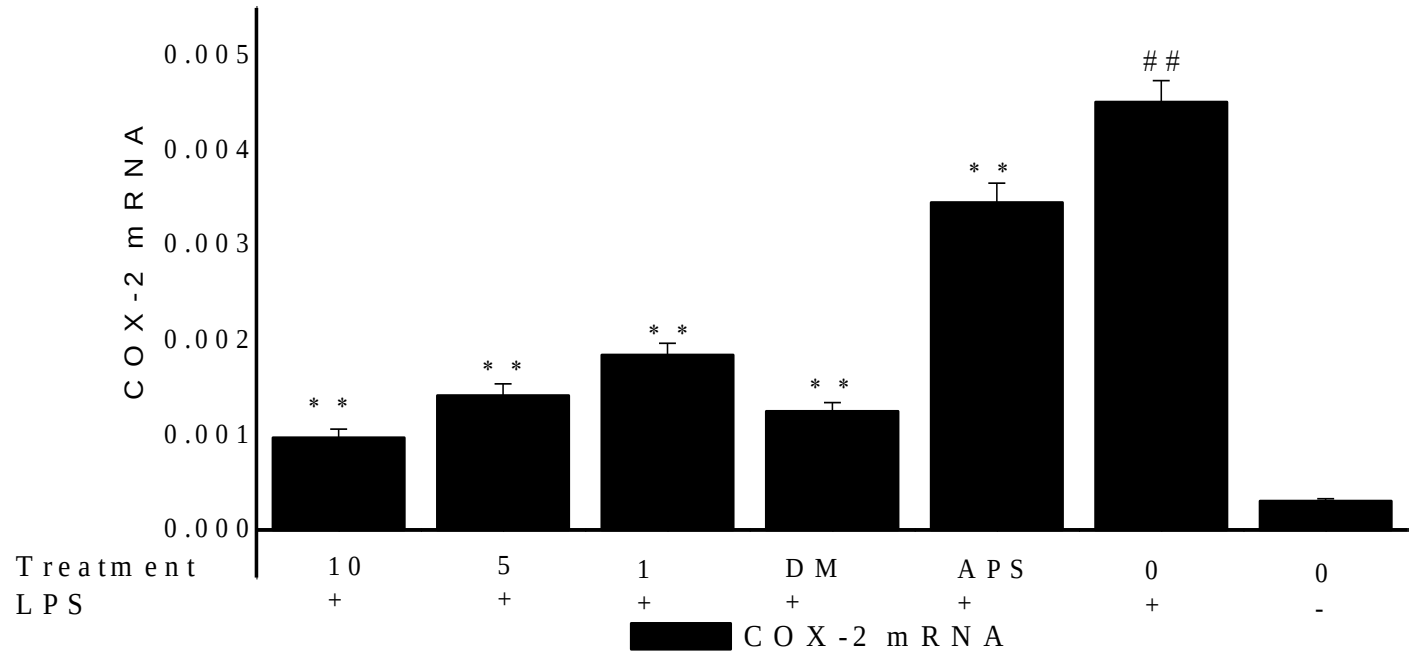


Figure 9. Effects of UA on COX-2 mRNA production. RAW264.7 cells were treated with LPS (10 ng/ml) in the presence of various concentrations of UA. COX-2 mRNA levels in the cell culture media by real-time PCR.
Data shown were the mean ± SD (n = 3). *p < 0.05; **p < 0.01 versus LPS alone, $^{\#}p$ < 0.05 or $^{\#\#}p$ < 0.01 compared to normal cell.

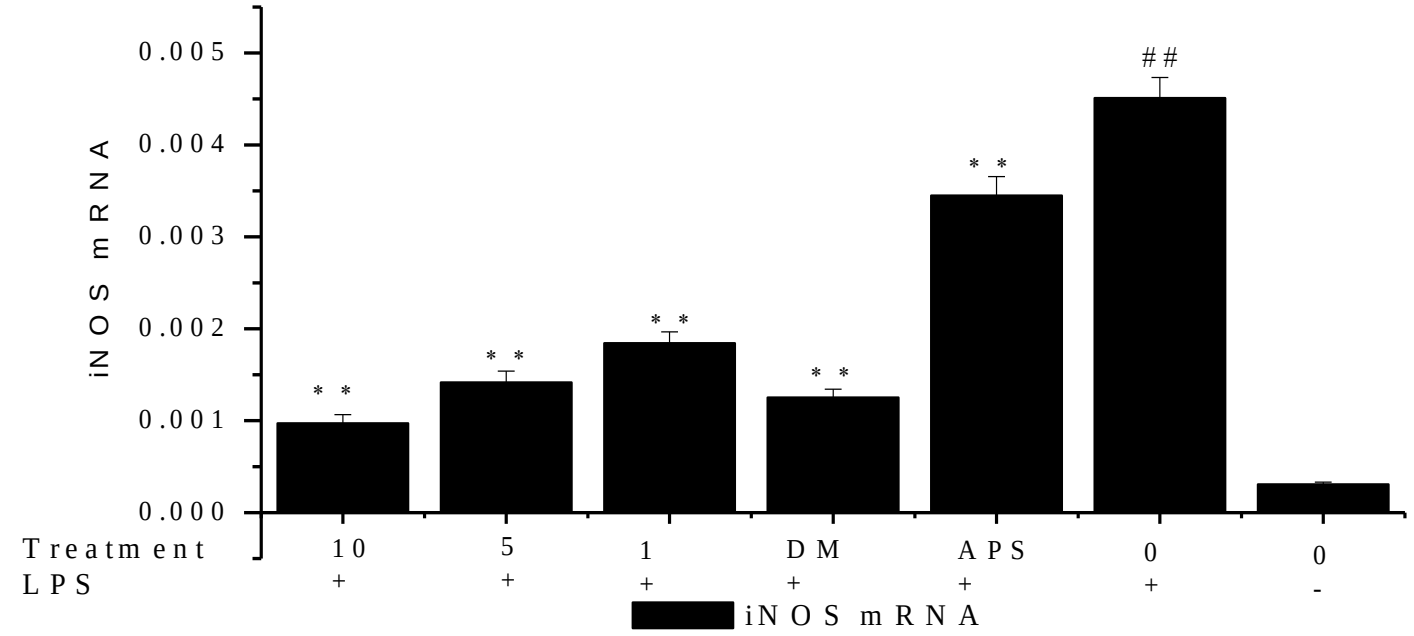


Figure 10. Effects of UA on iNOS mRNA production. RAW264.7 cells were treated with LPS (10ng/mL) in the presence of various concentrations of UA. iNOS mRNA levels in the cell culture media by real-time PCR.
Data shown were the mean ± SD (n= 3). *p < 0.05; **p < 0.01 versus LPS alone, $^{\#}p$<0.05 or $^{\#\#}p$ < 0.01 compared to normal cell.

results showed that UA could down-regulate the production of NF-κB, indicating that the inhibitory effects of UA on the production of NO, iNOS, COX-2, IL-1β, and TNF-α in LPS-stimulated RAW 264.7 cells might be

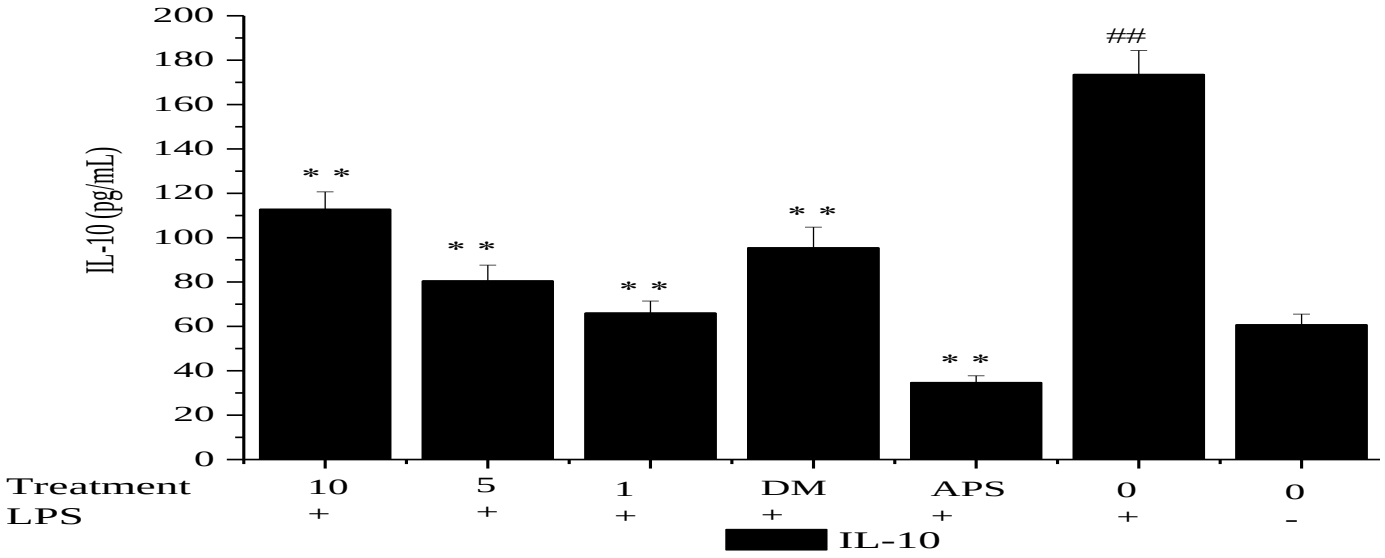


Figure 11. Effects of UA on IL-10 production. RAW264.7 cells were treated with LPS (10ng/mL) in the presence of various concentrations of UA. IL-10 levels were measured in the cell culture media by real-time PCR.
Data shown were the mean ±SD (n= 3). *p < 0.05; **p < 0.01 versus LPS alone, $^{\#}p$ < 0.05 or $^{\#\#}p$ < 0.01 compared to normal cell.

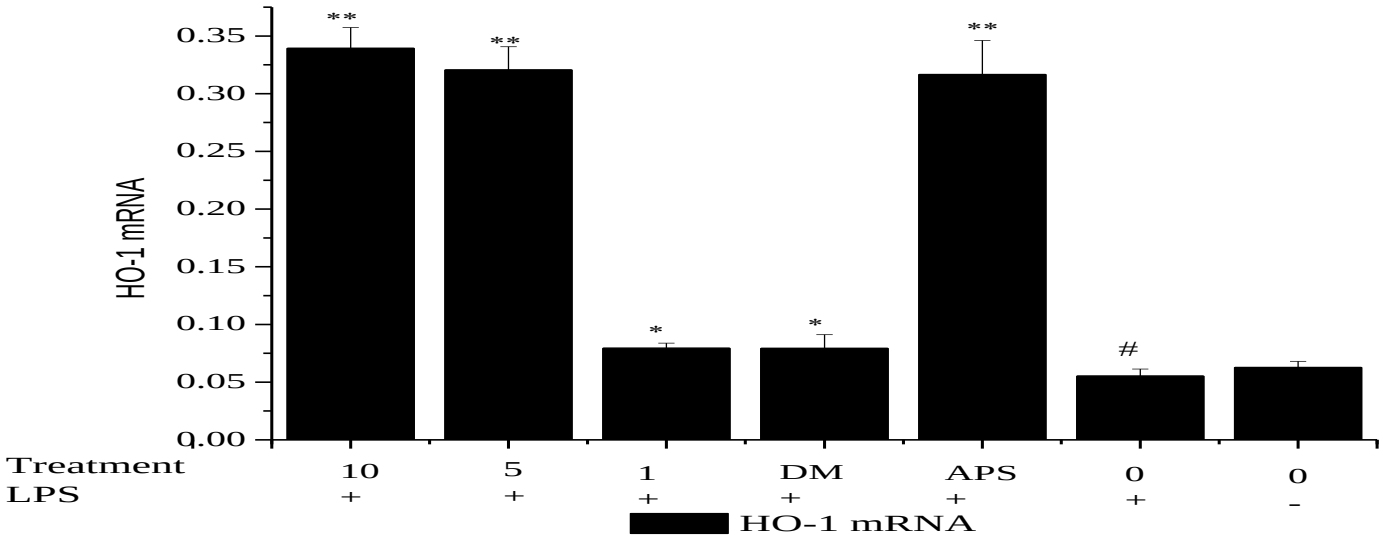


Figure 12. Effects of UA on HO-1 mRNA production. RAW264.7 cells were treated with LPS (10 ng/ml) in the presence of various concentrations of UA. HO-1 levels were measured in the cell culture media by real-time PCR.
Data shown were the mean ± SD (n = 3). *p < 0.05; **p < 0.01 versus LPS alone, $^{\#}p$ < 0.05 or $^{\#\#}p$ < 0.01 compared to normal cell.

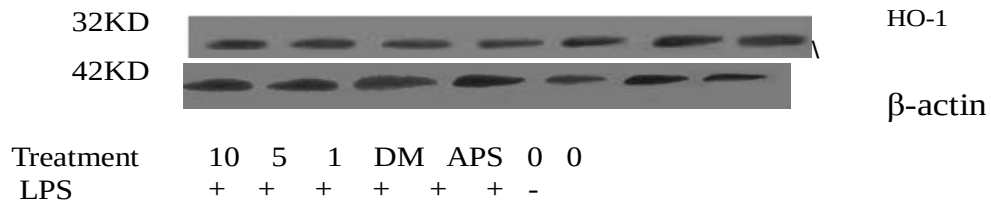


Figure 13. Effects of UA on HO-1 protein production. RAW264.7 cells were treated with LPS (10 ng/ml) in the presence of various concentrations of UA. HO-1 protein levels were measured in the cell culture media by western blot.

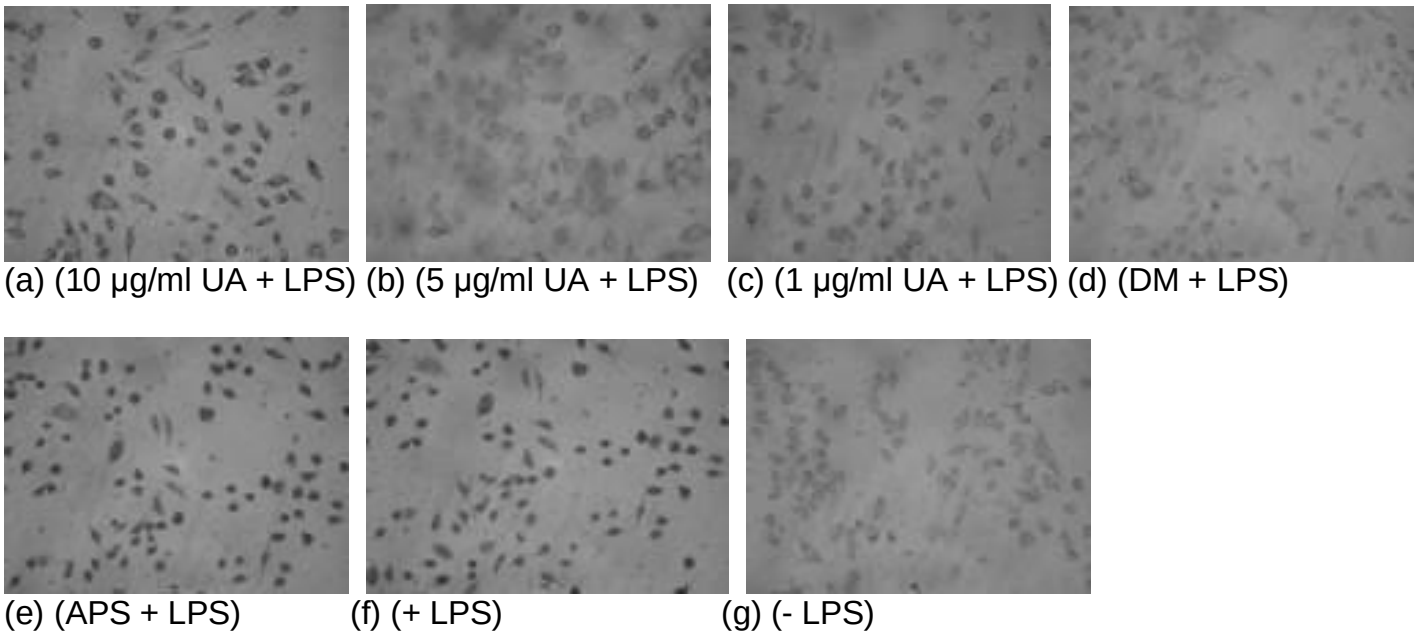


Figure 14. Effect of usnic acid (UA) on NF-κB in LPS-stimulated RAW 264.7 cell. RAW264.7 cells were treated with LPS (10 ng/ml) in the presence of various concentrations of UA. NF-κB levels were measured in the culture media of LPS-stimulated cells for 24 h by immunocytochemistry. The cells were colorized with diaminobenzidine, counterstaing with hematoxylin, ethanol dehydration (orderly by 75%, 95 and 100%). (1 × 40 times in microscope).
(a) High dose group, 10 μg/ml UA and 10 ng/ml LPS. (b) Middle dose group, 5 μg/ml UA and 10 ng/ml LPS. (c) Low dose group, 1 μg/ml UA and 10 ng/ml LPS. (d) Positive control, 0.5 μg/ml DM plus 10 ng/ml LPS. (e) Negative control, 100 μg/ml APS plus 10 ng/ml LPS. (f) Blank control, cells stimulated by 10 ng/ml LPS. (g) Normal control, cells incubated in 1640 medium without any drugs.

Conclusion

The molecular mechanisms on anti-inflammation of UA firstly isolated from *P. saxatilis* had been addressed in this study. The strong anti-inflammatory activity was down-regulating pro-inflammatory cytokines and mediators via suppressing NF-kB and up-regulating anti-inflammatory factors of IL-10 and HO-1. A further evaluation of UA on anti-inflammation is undergoing.

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REFERENCES

Baumann H, Gauldie J (1994). The acute phase response. Immunol. Today 15: 74- 80.
Bertolini A, Ottani A, Sandrini M (2001). Dual acting anti - inflammatory drugs: a reappraisal. Pharmacol. Res. 44(6):437-450.
Clancy RM, Amin AR, Abramson SB (1998). he role of nitric oxide in inflammation and immunity. Arthritis Rheum. 41(7):1141-1151.
Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. 126(1):131-138.
Hofman P (2004). Molecular regulation of neutrophil apoptosis and potential targets for therapeutic strategy against the inflammatory process. Curr. Drug Targets Inflamm. Allergy 3(1):1-9.
Ingolfsdotir K, Chung GAC, Skulason VG, Gissurarson SR, Vilhelmsdottir M (1998). Antimycobacterial activity of lichen metabolites *in vitro*. Eur. J. Pharm. Sci. 6(2):141-144.
Ingolfsdtir K. (2002). Usnic acid. Phytochemistry 61(7):729736.
Inui Y, Azuma Y, Ohura K (2002). Differential alteration of functions of rat peritoneal macrophages responsive to endogenous opioid peptide endomorphin-1. Int. Immunopharmacol. 2(8):1133-1142.
Li CQ. He LC. Jin JQ (2007). Atractylenolide I and atractylenolide III inhibit lipopolysaccharide-induced TNF-alpha and NO production in macrophages. Phytother Res. 21:347–353.
Kim KM, Kwon YG, Chung HT, Yun YG, Pae HO, Han JA. (2003). Methanol extract of Cordyceps pruinosa inhibits in vitro and in vivo inflammatory mediators by suppressing NF-kappaB activation. Toxicol. Appl. Pharmacol. 190(1):1-8.
Kim SS, Oh OJ, Min HY, Park EJ, Kim Y, Park HJ.(2003). Eugenol suppresses cyclooxygenase-2 expression in lipopolysaccharide-stimulated mouse macrophage RAW264.7 cells. Life Sci. 73:337-348.
Kröncke KD, Fehsel K, Kolb-Bachofen V (1998). Inducible nitric oxide synthase in human diseases. Clin. Exper. Immunol. 113:147-156.
Kumar KCS, Banskota AH, Manandhar MD (1996). Isolation and identification of some chemical constituents of Parmelia nepalensis. Planta Med. 62(1):93-94.
Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA (2001).

Possible new role for NF-kappaB in the resolution of inflammation. Nat. Med. 7(12):1291-1297.
Livak KJ, Schmittgen TD (2000). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25(4):402-408.
Makarov SS (2000). NF-kappaB as a therapeutic target in chronic inflammation: recent advances. Mol. Med. Today 6(11):441-448.
Mongan LC, Jones T, Patrick G (2000). Cytokine and free radical responses of alveolar macrophages in vitro to asbestos fibres. Cytokine 12(8):1243-1247.
Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65(1-2):55-63.
Naldini A, Carraro F (2005). Role of inflammatory mediators in angiogenesis. Curr. Drug Targets Inflamm. Allergy 4(1):3-8.
Ogborne RM, Rushworth SA, Charalambos CA, O'Connell MA (2004). Haem oxygenase-1: a target for dietary antioxidants. Biochem. Soc. Trans. 32(6):1003-1005.
Plutzky J (2001). Peroxisome proliferator-activated receptors in endothelial cell biology. Curr. Opin. Lipidol. 12:511–8.
Renard P, Raes M (1999). The proinflammatory transcription factor NFkappaB: a potential target for novel therapeutical strategies. Cell Biol. Toxicol. 15(6):341-344.
Riehemann K, Behnke B, Schulze-Osthoff K (1999). Plant extracts from stinging nettle (Urtica dioica), an antirheumatic remedy, inhibit the proinflammatory transcription factor NF-kappaB. FEBS Lett. 442:89-94.
Stadnyk AW, Gillan TL, Anderson R (1997). Respiratory syncytial virus triggers synthesis of IL-6 in BALB/c mouse alveolar macrophages in the absence of virus replication. Cell Immunol. 76:122-126.
Vartia KO (1973). Antibiotics in lichens. In: Ahmadjian V, Hale M E (Eds.). The Lichens. Academic Press, New York. P 547.
Zhao L, Tao JY, Zhang SL, Pang R, Jin F, Dong JH (2007). Inner anti-inflammatory mechanisms of petroleum ether extract from Melilotus *suaveolens* Ledeb. Inflammation 30(6):213-223.

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Full Length Research Paper

Light, temperature, and aging dependent vegetative growth and sporulation of *Colletotrichum gloeosporioides* on different culture media

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The fungal organism *Colletotrichum gloeosporioides* is the causative agent of anthracnose disease of *Citrus* fruits. It is recently introduced as a potential producer of anticancer metabolite paclitaxel. Here, we introduce the optimal conditions for growth and sporulation of *C. gloeosporioides*. We have considered four fungal culture media, that is potato dextrose agar (PDA), carnation leaf agar (CLA), potato carrot agar (PCA) and water agar (WA), based on which sporulation inducers like Watman or Fabriano filter papers could be added, and evaluated both for vegetative growth and sporulation. Three light regimens, i.e. continuous light, 16/8 hrs light/darkness, and continuous darkness were applied in combination with the culture media. All experiments were tracked on 7th, 15th, 21st, and 30th day after incubation. At 28°C, PDA and PCA culture media, under continuous light, provided the best condition for *C. gloeosporioides* maximal growth. Decreasing light periods decreased the fungal growth. Furthermore, fungal sporulation showed a high dependence on light, temperature and culture medium in use. Under 16/8 h light/darkness interval at the same temperature *C. gloeosporioides* sporulation was at its maximum on Fabriano paper placed on PDA medium. At a lower temperature, that is 22°C, *C. gloeosporioides* sporulation on the same culture media was highly defected. Furthermore, aging generally increased the fungal sporulation.

Key words: *Colletotrichum gloeosporioides*, citrus, growth, conidiation, development.

INTRODUCTION

The ascomycetous fungus *Colletotrichum,* members of which are anamorphic *Glomerella* species (Sutton, 1992; Armstrong-Cho and Banniza, 2006; Pfenning et al., 2007), is one of the most economically important complexes of plant pathogens, causing post-harvest rots, anthracnose, and blights of aerial plant parts. The symptoms typically appear as small to large, dark-colored spots or slightly sunken lesions on the foliage, stems or fruits of a wide range of tropical, subtropical and temperate crops (Bailey and Jeger, 1992). Although *Colletotrichum* species are classified as virulent pathogens, several species including *Colletotrichum gloeosporioides* can express mutualistic lifestyles in non-disease hosts (Rodriguez and Redman, 2008). Individual isolates of *Colletotrichum* species can express either parasitic or mutualistic lifestyles depending on the host genotype colonized. Mutualistic benefits of *Colletotrichum* spp. to hosts include growth enhancement, disease

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resistance, and/or abiotic stress tolerance (Redman et al., 2001).

Virulent *Colletotrichum* species employ a hemibiotrophic strategy to invade host, in which biotrophic and necrotrophic stages of infection are sequentially established (Perfect et al., 1999). Upon germination of conidia of these fungi, the hyphae penetrate the host cell lumen through host cuticle and cell. These intracellular hyphae are biotrophic (O'Connell et al., 2004). Afterwards, necrotrophic hyphae forms, extensively spread and kills the host tissue.

Colletotrichum species, compared to obligate biotrophs, can be cultured *in vitro*. They are amenable to genetic transformation by a variety of methods, especially *Agrobacterium*-mediated transformation (de Groot et al., 1998; Tsuji et al., 2003; Takahara et al., 2004; Flowers and Vaillancourt, 2005; Talhinhas et al., 2008; Ushimaru et al., 2010; Nakamura et al., 2012; Yousefi-Pour Haghighi, 2013). Since *Colletotrichum* species are haploid, molecular genetics approaches are facile in these fungi. Hence, they have been served as excellent models for the study of fungal morphogenesis and pathogenicity.

The phytopathogenic fungus *C. gloeosporioides* (Penz) Penz & Sacc in Penz, (Teleomorph: *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk), causes anthracnose on many tropical, subtropical and temperate fruits (Waller, 1992), especially on *Citrus* species. Post-harvest problems caused by *C. gloeosporioides* especially in the tropics, often are a significant factor in limiting export (Fitzell and Peak, 1984). The economic cost of cryptic infections caused by *C. gloeosporioides* is about 25% greater than that for field losses (Jeger and Plumbley, 1988). These have grouped *C. gloeosporioides* among the most important post-harvest pathogens. In addition to its considerable detrimental economic importance, it has been shown that endophytic, apparently nonpathogenic, *Colletotrichum* species including *C. gloeosporioides* are a source of secondary metabolites with anticancer effects, that is paclitaxel (Taxol) (Gangadevi and Muthumary, 2008; Strobel et al., 1999).

C. gloeosporioides sensu lato is a species complex with broad genetic and biological diversity. It is associated with at least 470 different host genera (Sutton, 1980). Several cultural and environmental factors affecting the growth of this species, *in vitro*, are studied in isolates from different host genera including papaya, green pepper and *Plumeria* (Nithya and Muthumary, 2009; Silveira et al., 2004). Because of the economically significance of *C. gloeosporioides* for *Citrus* species (Adaskaveg and Förster, 2000; Ramos et al., 2006) and its significance for fermentation-based paclitaxel production (Gangadevi and Muthumary, 2008; Strobel et al., 1999), here we aimed at quantifying and comparing vegetative mycelial growth and sporulation of a *Citrus* isolate of this fungus on twelve synthetic culture media, under different light and temperatures regimens.

MATERIALS AND METHODS

Fungal strain

Colletotrichum gloeosporioides wildtype strain JS-1389 (obtained from National Plant Protection Institute, Tehran, Iran), which was isolated as a plant pathogen from *Citrus* species in Iran, was used as the model. The fungal strain was grown on potato dextrose agar (PDA) medium (purchased from Merck, Darmstadt, Germany) at 28°C. For long term usage, the fungus was maintained under liquid paraffin at 4°C.

Culture media

Four standard fungal solid culture media that is, PDA, potato carrot agar (PCA), carnation leaf agar (CLA) and water agar (WA) were employed for developmental studies (purchased from Merck, Darmstadt, Germany). In combination with those culture media, two kinds of filter papers, that is Watman No. 41 and Fabriano No. 808, in total 12 synthetic culture media, were investigated. All experiments were performed in three replicates.

Light and temperature regimens

The effects of two light conditions, that is 24 h light versus 8 h dark/16 h light; as well as two temperatures, i.e. 22 and 28°C on fungal development were evaluated in combination with culture media which had shown supportive effect for fungal development.

Assessment of fungal development

Mycelial discs of 7 mm diameter cut from the growing margins of the fresh fungal culture were placed at the center of each 9 cm Petri plate. Two factors, that is diameter of mycelial growth (mm), and sporulation of the fungus (× 10) under different regimens were measured on a daily (4, 10, 15, 20, 30) interval.

Statistical analyses

Analysis of variance (ANOVA) and SAS procedures and programs were used for statistical analyses. In cases where the F-test showed significant differences among means, the differences among treatments were compared using least significant differences (LSD) test at 1% significance level (Steel et al., 1997). In cases where there was zero number, like absence of sporulation, the non-parametric statistics and Wilcokson's test were applied.

RESULTS

Fungal mycelia growth under 24 h light at 28°C on different culture media

At five daily intervals, that is 4th, 10th, 15th, 20th, and 30th days, fungal growth was measured under continuous light on solid culture media. Significant differences were observed for mycelial growth under 24 h light at 28°C, using different culture media, and filter papers ($P \leq 0.01$; not shown). Table 1.

The interaction of culture media and filter papers was also statistically significant ($P \leq 0.01$) for mycelia growth.

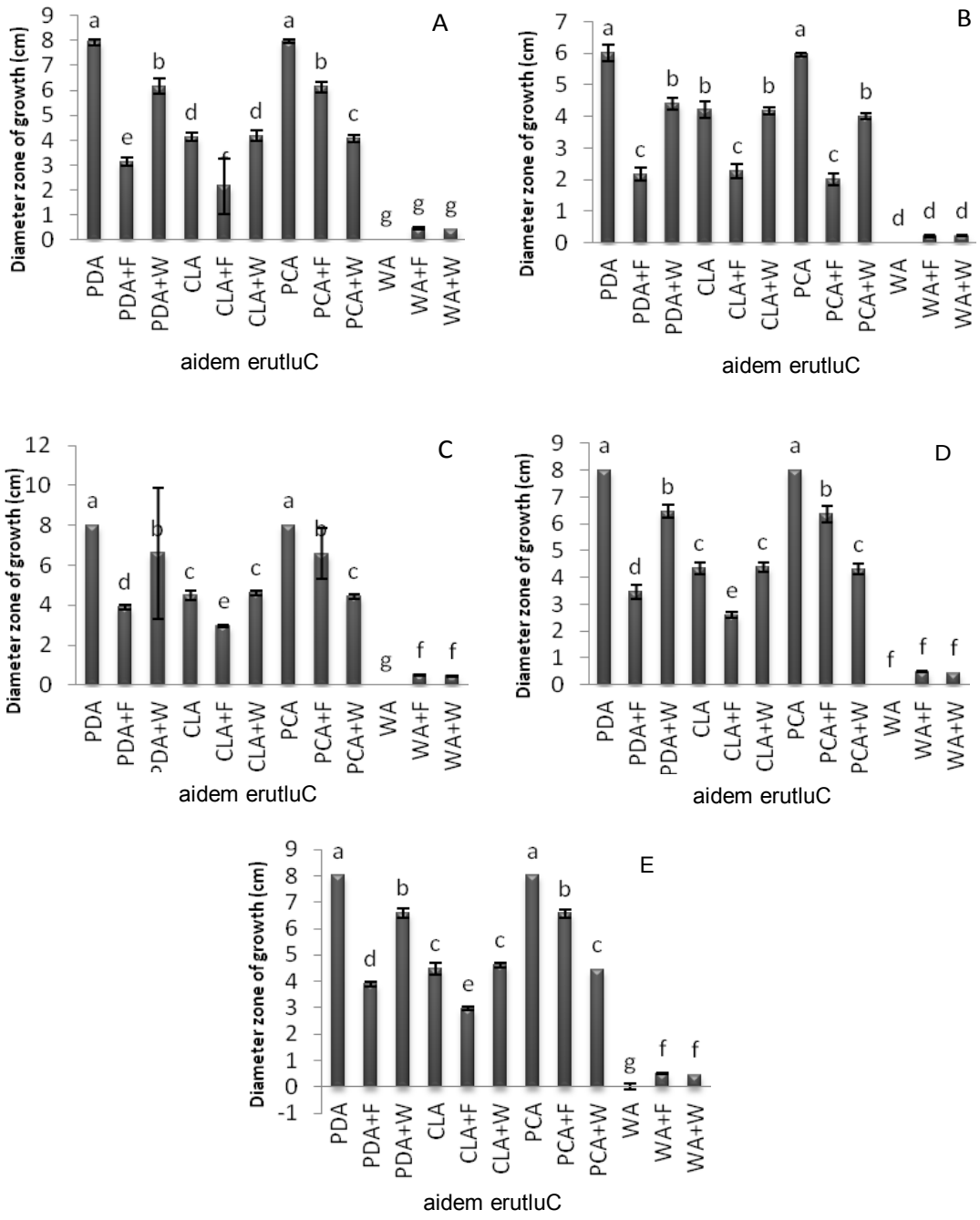

Figure 1. Mycelial growth of *C. gloeosporioides* on 12 culture media under 24 h light at 28 to 30°C on daily intervals. (A) 4th, (B) 10th, (C) 15th, (D) 20th, (E) 30th day. Data (significant at $P \leq 0.01$) are averages of three replicates. Error bars indicate standard errors. Similar letters indicate no significant difference.

As seen in Figure 1, under 24 h light, the best culture media for fungal mycelia growth were PDA and PCA, on which *C. gloeosporioides* JS-1389 could establish itself over 4 days incubation, and after 10 days fungus had covered the whole Petri plate. Among other culture media tested, PDA + Watman filter and PCA + Fabriano filter better favored fungus growth, but it took almost 20 days for fungus to establish its colony. The fungus could grow on CLA-based culture media over 4 days, but hardly could grow further. It seems that on PDA- and CLA-based media the growth induction effect of Watman filter was more than Fabriano filter. However, on PCA-based media the opposite effect was seen. WA-based culture media provided the worst condition for fungal mycelia growth

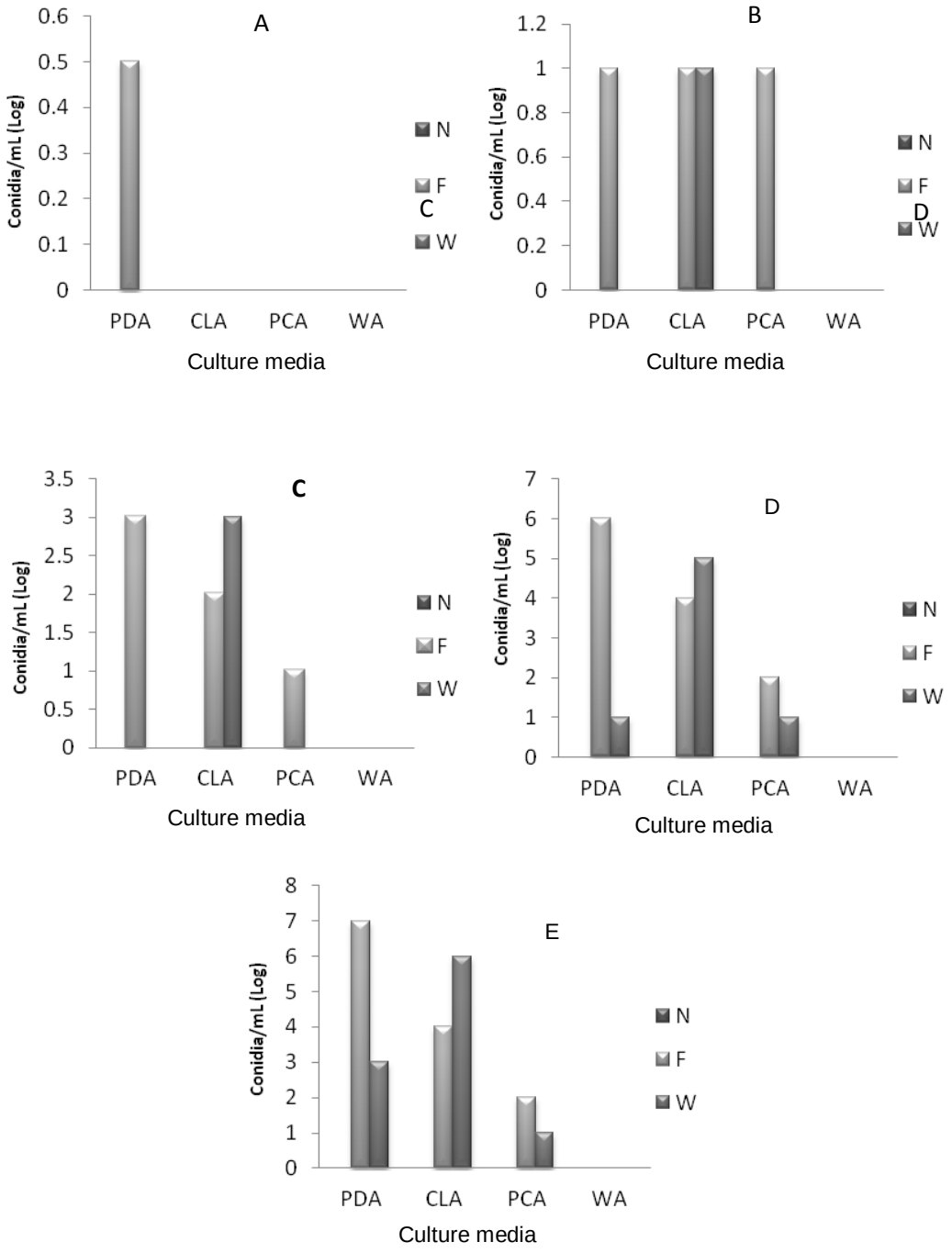

Figure 2. Sporulation concentration (spore ml^{-1}) of *C. gloeosporioides* on 12 culture media under 24 h light at 28 to 30°C on daily intervals. (A) 4th, (B) 10th, (C) 15th, (D) 20th, (E) 30th day.
Data (significant at P ≤ 0.01) are averages of three replicates.

growth under continuous light. Including filter papers on such media improved fungal growth.

Fungal sporulation under 24 h light at 28°C on different culture media

At five daily intervals, that is 4th, 10th, 15th, 20th, and 30th days, fungal sporulation was measured under continuously light on solid culture media. Significant differences were observed for sporulation under 24 h light at 28°C, using different culture media, and filter papers (P ≤ 0.01; not shown) Table 2. The interaction of culture media and filter papers was also statistically significant (P ≤ 0.01). As seen in Figure 2, under 24 h light the best culture

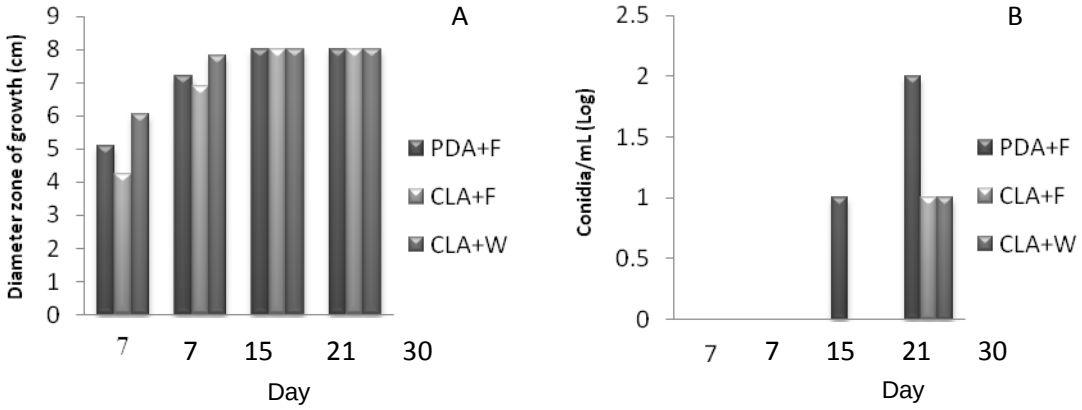

Figure 3. *C. gloeosporioides* mycelial growth (A) and sporulation (B) under 24 h darkness at 28 to 30°C on three different culture media.
Data are averages of two replicates.

medium for fungal sporulation was PDA + Fabriano filter, on which sporulation started as early as 4 days, and continuously raised up to × 10^7 conidia per ml on 30th day. Moreover, after 10 days, *C. gloeosporioides* JS-1389 could sporulate also on CLA + Whatman filter, CLA + Fabriano filter and PCA + Fabriano filter, but in the range of 10 conidia per ml. Afterwards, filter paper containing CLA media favored fungal sporulation, up to × 10^6 conidia per ml on 30th day. However, filter paper containing PCA media hardly favored further fungal sporulation. Notably, without providing filter papers, the fungus could sporulate neither on WA-based media, nor on PDA, CLA, and PCA.

Fungal mycelia growth and sporulation under 24 h darkness at 28°C on different culture media

At four daily intervals, that is 7th, 15th, 21th, and 30th days, fungal development was measured under darkness on three solid culture media which had shown the best effects on fungal mycelia growth and sporulation, that is PDA + Fabriano paper, CLA + Fabriano paper, and CLA + Whatman paper (Figures 1 and 2). As indicated in Figure 3A and B, significant differences were observed for sporulation under 24 h darkness at 28°C, using different culture media and filter papers. As seen in Figure 3A, until day 15, the *C. gloeosporioides* JS-1389 mycelia growth was better on CLA + Whatman paper, than two other media, under continuous light at 28°C. On this culture medium, the fungus had covered the whole Petri plate after 15 days. However, the difference was not so significant, as on day 21 the fungus had covered the whole Petri plate on all three culture media. As seen in Figure 3B, under continuous light, on neither culture media *C. gloeosporioides* JS-1389 could sporulate over 15 days. However, as shown, on PDA + Fabriano paper up to 10 per ml conidia was produced on 21th day, and up to × 10^2 on 30th day. However, after 30 days, the fungus could also sporulate on CLA + Fabriano paper, and CLA + Whatman paper under continuous light at 28°C, albeit about 10-fold less than on PDA + Fabriano paper. This indicates that aging also improved the fungal sporulation under this condition.

Fungal mycelia growth and sporulation under 16/8 h light/darkness at 28 and 22°C on different culture media

At four daily intervals, that is 7, 15, 21, 30th days, *C. gloeosporioides* JS-1389 development was measured under ligh/darkness on three solid culture media which had shown the best effects on fungal development, that is PDA + Fabriano paper, CLA + Fabriano paper, and CLA + watman paper. Here, two temperatures for incubation period were applied, that is 28 and 22°C. As indicated in Figure 4A and B, and in Figure 5A and B, significant differences were observed for sporulation under 16/8 light/darkness h at 28 and 22°C, using different culture media, and filter papers. As it is shown in Figure 4A, at 28°C under 16/8 h light/darkness, mycelia growth was almost identical on all three culture media. On day 21, *C. gloeosporioides* JS-1389 could cover the whole Petri plate on all media. However, as shown in Figure 4B, under this condition the maximum sporulation was appeared on PDA + Fabriano paper medium. It is seen that this medium favored fungal sporulation, ~ × 10^4 conidia per ml on 15th day, which rose to ~ × 10^6 on 21th day, and ~ × 10^8 conidia per ml on 30th day. This suggests that aging also improved the fungal sporulation under this condition. On both CLA + Fabriano paper and CLA + Whatman paper media, the fungus could only sporulate ~ × 10^5 conidia per ml on 30th day. As it is shown in Figure 5A, at 22°C, mycelia growth under 16/8 h light/darkness did not depend on culture media. On day

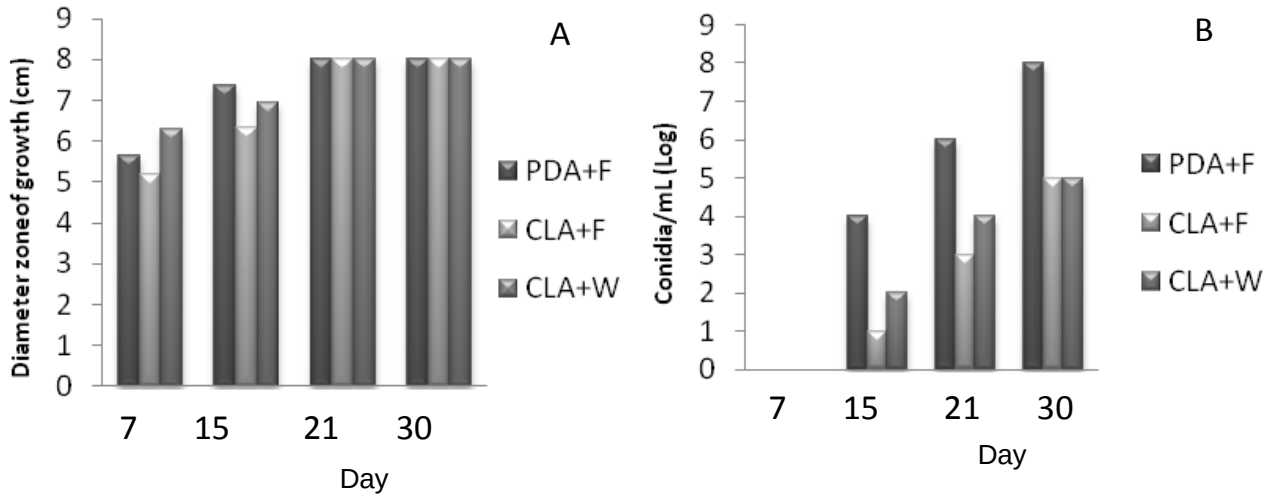

Figure 4. *C. gloeosporioides* mycelial growth (A) and sporulation (B) under 16/8 h light/darkness at 28 to 30°C on different culture media on three different culture media. Data are averages of two replicates.

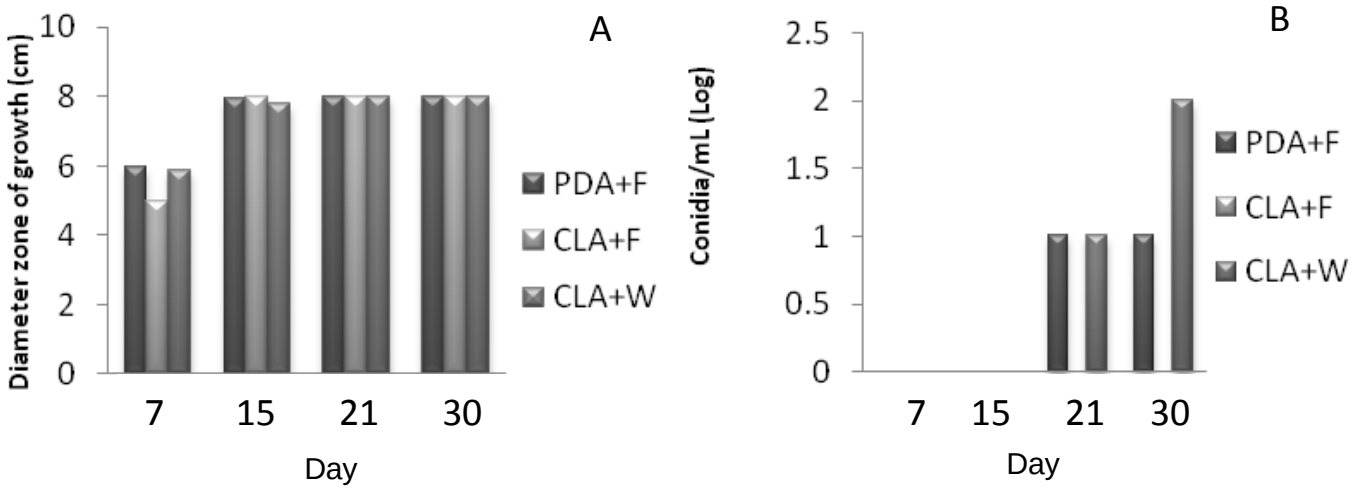

Figure 5. *C. gloeosporioides* mycelial growth (A) and sporulation (B) under 16/8 h light/darkness at 22°C on different culture media on three different culture media.
Data are averages of two replicates.

15th, *C. gloeosporioides* JS-1389 could cover the whole Petri plate on all three media. However, as shown in Figure 5B, under this condition the maximum sporulation was appeared on CLA + Whatman paper medium, only on 30th day, and maximally ~ × 10^2 conidia per ml. This was 10-fold less on PDA + Fabriano paper and CLA + Fabriano paper media.

DISCUSSION

C. gloeosporioides is currently a fungal model for plant-microbe interaction studies, as well as, a microbial source for anticancer drug fermentation. At fungal natural niches, light and nutrients are among the most environmental factors affecting the success and fitness of the fungus. However, for a better handling of the fungus under laboratory conditions there is a need for understanding its development that is vegetative growth and sporulation. Several researches have introduced a number of mixed solid media for gaining the maximal colonization of the fungus isolated from different hosts, and to obtain enough conidia for further experiments. We initially aimed at using a Citrus isolate of *C. gloeosporioides* for functional genetic studies in our laboratory, but by using the formerly introduced culture media we could not obtain sufficient

Table 1. Analysis of variance and means comparison for *C. gloeosporioides* mycelial growth under 24 h light at 28 to 30°C.

Parameter	No.	Mean square (Mycelial growth cm)				
S.O.V	F	4th day	10th day	15th day	20th day	30th day
Filter paper	2	17.45**	12.75**	10.59**	8.32**	8.32**
Media	3	32.53**	63.77**	67.68**	70.71**	70.71**
F×M	6	3.06**	6.82**	6.33**	5.69**	5.69**
Error	24	0.027**	0.029**	0.029**	0.012**	0.012**
CV%	-	5.54	4.39	4.22	2.6	2.6

**Significant at 1% level.

Table 2. Analysis of variance and means comparison for *C. gloeosporioides* sporulation under 24 hrs light at 28 to 30ºC by the non-parametric statistics and Wilcokson's test.

Parameter	Mean square (Sporulation spores/ml)				
S.O.V	4th day	10th day	15th day	21th day	30th day
Filter paper × Media	10^{1}*	10^{5}*	10^{4}*	10^{7}*	10^{7}*

**Significant at 5% level

sufficient conidia. Hence, we tried first to find the optimized condition in which *C. gloeosporioides* grows and sporulates best. We first considered four basic fungal culture media, i.e. PDA, CLA, PCA and WA, based on which sporulation inducers like filter papers could be added (in total twelve culture media), and evaluated both for vegetative growth and sporulation. Three light regimens that is, continuous light, 16/8 h light/ darkness, and continuous darkness were applied in combination with three top culture media. *C. gloeosporioides* mycelia growth and sporulation were measured at five daily intervals, that is 4th, 10th, 15th, 20th, and 30th days. However, because of the improving effects of 16/8 h light/ darkness on fungal development at 28°C, this condition was also applied at 22°C.

As the initiative experiments showed, under continuous light at 28°C, PDA and PCA culture media provided the best media for *C. gloeosporioides* maximal mycelia growth. However, under this condition, the maximum sporulation was observed on PDA + Fabriano paper medium followed by CLA + Whatman paper and CLA + Fabriano paper media. We then evaluated *C. gloeosporioides* mycelial growth and sporulation on those three culture media under continuous darkness at 28°C. Under this condition the growth rate was decreased. Notably, the sporulation of the fungus was defected. This indicates the significance of light for *C. gloeosporioides* vegetative growth and sporulation, independent of the culture media in use. Furthermore, under alteration of 16/8 h light/ darkness at 28°C, *C. gloeosporioides* vegetative growth was decreased compared to continuous light, and the growth was almost identical to continuous darkness. However, the maximum sporulation of the fungus was observed at this situation. This indicates that 16/8 h light/darkness intervals at the same temperature improve *C. gloeosporioides* sporulation compared to both continuous light and continuous darkness. We then evaluated fungal development under 16/8 h light/darkness at 22°C on the same culture media. *C. gloeosporioides* vegetative growth was almost identical, but the sporulation of the fungus was highly decreased at 22°C compared to that of 28°C. This indicates that sporulation of *C. gloeosporioides* is significantly temperature-dependent (Figure 6).

Taking all together, our data indicate that at 28 to 30°C, PDA and PCA culture media provide the best condition for *C. gloeosporioides* maximal growth, especially under continuous light. Shortage

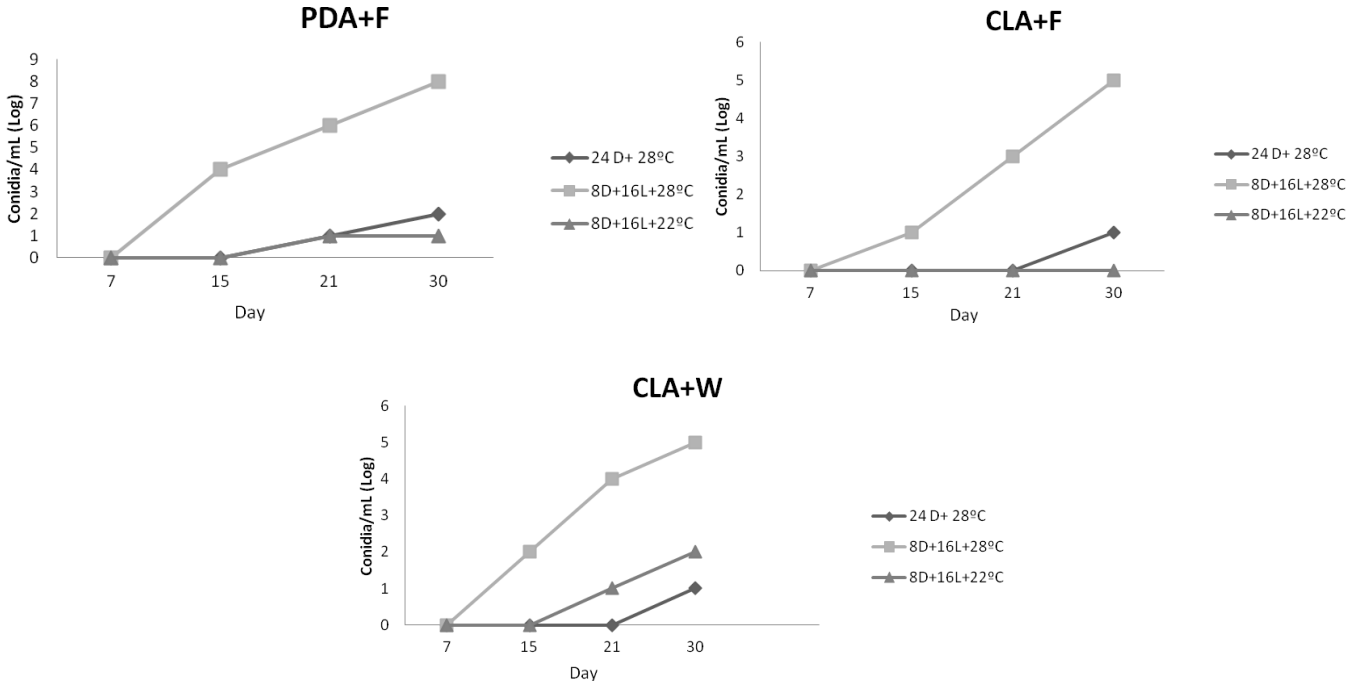

Figure 6. Comparison of sporulation of *C. gloeosporioides* on PDA+F, CLA+F, and CLA-W culture media, under three different light and temperature regimens, at four daily intervals.

of light could decrease the fungal growth. So, *C. gloeosporioides* vegetative growth is light dependent. Furthermore, our data suggest that fungal sporulation is highly light- , temperature- and culture medium-dependent. Indeed, under 16/8 h light/darkness intervals at the same temperature *C. gloeosporioides* sporulation was at its maximum on PDA + Fabriano paper medium, compared to its sporulation under both continuous light and continuous darkness. This suggests that both light alteration and culture media influence fungal sporulation. Moreover, decreasing incubation temperature to 22°C, highly decreases *C. gloeosporioides* sporulation on the same culture media. It should also be noted that in all experiments it was obvious that aging increased the fungal sporulation.

Fungal vegetative growth on nine different culture media under three different light regimens was almost identical. Indeed, the fungus could grow on all the culture media tested, regardless of light conditions. This means that mycelia growth was independent of light; although an alternation of 16/8 h light/darkness improved the fungal vegetative growth compared to either continuous light or continuous darkness. In total, the PDA culture medium could provide the best condition for *Piriculaiia oryzae* vegetative growth, regardless of light.

ACKNOWLEDGEMENT

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REFERENCES

Adaskaveg JE, Förster H (2000). Occurrence and management of anthracnose epidemics caused by *Colletotrichum* species on tree fruit crops in California. In: Prusky D, Freeman S, Dickman MB (eds.), *Colletotrichum*. Host Specificity, Pathology and Host-Pathogen Interaction. APS Press, St Paul, Minnesota. pp. 317-336.

Armstrong-Cho CL, Banniza S (2006). *Glomerella truncata* sp. nov., the teleomorph of *Colletotrichum truncatum*. Mycol. Res. 110:951-956.

Bailey JA, Jeger MJ (1992). *Colletrotrichum*: biology, pathology and control. CAB International, Wallingford, UK. P 388.

de Groot MJ, Bundock P, Hooykaas PJJ, Beijersbergen AG (1998). *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat. Biotechnol. 16:839-842.

Fitzell RD, Peak CM (1984). The epidemiology of anthracnose disease of mango: inoculum sources, spore production and dispersal. Ann. Appl. Biol. 104:53–59.

Flowers JL, Vaillancourt LJ (2005). Parameters affecting the efficiency of *Agrobacterium tumefaciens*-mediated transformation of *Colletotrichum graminicola*. Cur. Genet. 48:380-388.

Gangadevi V, Muthumary J (2008). Isolation of *Colletotrichum gloeosporioides,* a novel endophytic taxol-producing fungus from the leaves of a medicinal plant. *Justicia gendarussa*. Mycol. Balcanica 5:1–4.

Jeger MJ, Plumbley RA (1988). Post-harvest losses caused by anthracnose (*Colletotrichum gloeosporioides*) of tropical fruits and vegetables. Biodeterioration 7:642–646.

Nithya K, Muthumary J (2009). Growth studies of *Colletotrichum gloeosporioides* (Penz.) Sacc. - a taxol producing endophytic fungus from *Plumeria acutifolia*. Ind. J. Sci. Technol. 6:14-19.

O'Connell R, Herbert C, Sreenivasaprasad S, Khatib M, Esquerré-Tugayé MT, Dumas B (2004). A novel *Arabidopsis-Colletotrichum*

pathosystem for the molecular dissection of plant fungal interactions. Mol. Plant-Microbe Interact. 17:272-282.
Perfect SE, Hughes HB, O'Connell RJ, Green JR (1999). *Colletotrichum*: a model genus for studies on pathology and fungal–plant interactions. Fungal Genet. Biol. 27:186–198.
Pfenning LH, Salgado M, Lima CS (2007). New concepts in the systematics of *Colletotrichum*. Fitopatol. Brasileira 32:20-21.
Ramos AP, Merali Z, Talhinhas P, Sreenivasaprasad S, Oliveira H (2006). Molecular and morphological characterization of *Colletotrichum* species involved in citrus anthracnose in Portugal. Bul. OILB/SROP 29:317-326.
Redman RS, Dunigan DD, Rodriguez RJ (2001). Fungal symbiosis: from mutualism to parasitism, who controls the outcome, host or invader? New Phytol. 151:705–716.
Rodriguez R, Redman R (2008). More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. J. Exp. Bot. 59:1109–1114.
Silveira MAF, Zamboni MAC, Bedendo IP (2004). Development of *Colletotrichum gloeosporioides* isolated from green pepper in different culture media, temperatures, and light regimes. Sci. Agricol. 61:542-544.
Steel RGD, Torrie JH, Dicky DA (1997). Principles and Procedures of Statistics –A Biometrical Approach, 3rd ed. McGraw Hill Book International Co., Singapore pp. 204–227.
Strobel GA, Ford E, Li JY, Sears J, Sidhu RS, Hess WM (1999). *Seimatoantlerium tepuiense* gen. nov., a unique epiphytic fungus producing taxol from the Venezuelan-Guayana. System Appl. Microbiol. 22:426-433.
Sutton BC (1980). The coelomycetes. Fungi imperfecti with pycnidia, acervuli and stromata. Commonwealth Mycological Institute, Kew, England.
Sutton BC (1992). The genus *Glomerella* and its anamorph *Colletotrichum*. In: Bailey JA, Jeger MJ (eds.), *Colletotrichum*: biology, pathology and control). CAB International: Wallingford. pp. 1-26.
Takahara H, Tsuji G, Kubo Y, Yamamoto M, Toyoda K, Inagaki Y, Ichinose Y, Shiraishi T (2004). *Agrobacterium tumefaciens*-mediated transformation as a tool for random mutagenesis of *Colletotrichum trifolii*. J. Gen. Plant Pathol. 70:93–96.
Talhinhas P, Muthumeenakshi S, Neves-Martins J, Oliveira H, Sreenivasaprasad S (2008). *Agrobacterium*-mediated transformation and insertional mutagenesis in *Colletotrichum acutatum* for investigating varied pathogenicity lifestyles. Mol. Biotechnol. 39:57–67.
Tsuji G, Fujii S, Fujihara N, Hirose C, Tsuge S, Shiraishi T, Kubo Y (2003). *Agrobacterium tumefaciens*-mediated transformation for random insertional mutagenesis in *Colletotrichum lagenarium*. J. Gen. Plant Pathol. 69:230-239.
Ushimaru T, Terada H, Tsuboi K, Kogou Y, Sakaguchi A, Tsuji G, Kubo Y. (2010). Development of an efficient gene targeting systein *Colletotrichum higginsianum* using a nonhomologous end-joining mutant and *Agrobacterium tumefaciens*-mediated gene transfer. Mol. Genet. Genom. 284:357–371.
Waller JM (1992). *Colletotrichum* disease of perennial and other cash crops In: Bailey JA, Jeger MJ (eds.), *Colletotrichum*: biology, pathology and control). CAB International: Wallingford. pp. 167–185.
Yousefi-Pour Haghighi M, Soltani J, Nazeri S (2013). A survey on optimization of *Agrobacterium*-mediated genetic transformation of the fungus *Colletotrichum gloeosporioides*. J. Cell. Mol. Res. 5:35-41.

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Full Length Research Paper

Standardization of Roselle (*Hibiscus sabdariffa* L.) Calyx cultivated in Sudan

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Herbal products have become increasingly popular throughout the world; one of the impediments in its acceptance is the lack of standard quality control profile. This research was conducted to standardize Roselle (*Hibiscus sabdariffa*) cultivated in Sudan. For this purpose, world health organization (WHO) monograph for medicinal plants was used to assess certain particulars of the plant including macro and microscopical characters, physical and chemical characteristics. Results obtained revealed that the macro and microscopical characters of *H. sabdariffa* were in accordance to those specified in WHO monographs. Results of purity tests were obtained as (16.3) water soluble extractive value (21.4%), 70% ethanol soluble extractive, (11.7%) the total ash, (3.8%) acid insoluble ash values and (8.5%) moisture content, respectively whereas value of foreign materials were calculated to be 2%. The heavy metal lead in the plant sample tested was found to be below the permissible limit (10 mg/kg) and the plant was found to be free from bacterial contamination. The high performance liquid chromatography (HPLC) analysis of *H. sabdariffa* revealed the presence of quercetin as a major phytoconstituent in a total content of 12.96%. It can be concluded that the obtained data of *H. sabdariffa* could be taken as guidelines in its quality assessment and will contribute positively to establish standards for the quality assessment of herbal products in Sudan.

Key words: Standardization, herbal medicine, *Hibiscus sabdariffa*.

INTRODUCTION

In recent years, plant derived products are increasingly being sought out as medicinal products, nutraceuticals and cosmetics and are available in health food shops and pharmacies over the counter as self-medication or also as drugs prescribed in the non-allopathic systems (Neeraj and Bhupinder, 2011). According to an estimate of the World Health Organization (WHO), about 80% of the world population still uses herbs and other traditional medicines for their primary health care needs (Amit et al., 2007). Herbal formulations have reached widespread acceptability as therapeutic agents for diabetics, arthritics, liver diseases, cough remedies, memory enhancers and adoptogens (Patel et al., 2006). Herbals are traditionally considered harmless and increasingly being consumed by people without prescription. How-ever, some can cause health problems, some are not effective and some may interact with other drugs. Stan-dardization of herbal formulations is essential in order to assess the quality of drugs, based on the concentration of their active principles (Yadav and Dixit, 2008). Quality evaluation of herbal preparation is a fundamental requirement of industry and other organization dealing with herbal products.

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The growing use of botanicals by the public is forcing moves to assess the health claims of these agents and to develop standards of quality and manufacture (Neeraj and Bhupinder, 2011). Standardization of herbal medicines is the process of prescribing a set of standards or inherent characteristics, constant parameters, definetive qualitative and quantitative values that carries an assurance of quality, efficacy, safety and reproducibility (Kunle et al., 2012).

Roselle (*H. sabdariffa*) is an edible plant used in various applications including foods. The fleshy red calyces are used for making wine, juice, jam, syrup, pudding, cakes, ice cream or herbal tea. Roselle flowers and calyces are also known for their antiseptic, diuretic, antioxidant and antimutagenic properties (Ali-Bradeldin et al., 2005). Roselle is an important source of vitamins, minerals, and bioactive compounds, such as organic acids, phytosterols and polyphenols, some of them with antioxidant properties. The phenolic content in the plant consists mainly of anthocyanins like delphinidin-3-glucoside, sambubioside and cyanidin-3-sambubioside; other flavonoids like gossypetin, hibiscetin, and their respective glycosides; protocatechuic acid, eugenol, and sterols like β-sitoesterol and ergoesterol (Azza et al., 2011). Hence, this study was undertaken to standardize the popular and widely used plant *H. sabdariffa*.

MATERIALS AND METHODS

H. sabdariffa calyces were purchased from the suppliers of natural remedies in Khartoum North Province during November, 2005.

Macroscopical characters of the plant

The authentication and macroscopical characters of the plant had been performed at the Medicinal and Aromatic Plant Research Institute (MAPRI), National Centre for Research (NCR), Khartoum, Sudan and Department of Botany, Faculty of Science, University of Khartoum.

Microscopical characters of the plant

The Permanent slides (Transverse sections (TS), Horizontal section (HS), were prepared according to Alexander (1940). Slides sample taken were prepared from soft tissue of *H. sabdariffa* and subjected to the following treatments. Preliminary treatment of the plant material was by using the standard fixative (FAA), formaldehyde was glacial acetic acid: 70% ethyl alcohol (5:5: 90 v/v). A mixture of 1:1 (cedar wood oil: absolute alcohol), pure cedar wood oil, a mixture of cedar wood oil and xylene, pure xylene. Sectioning: Soft tissues were sectioned using a rotary microtome (Leitz 1512 Germany), adjusted at 12 microns using a brush. Staining: by using pure xylene, safranin stain dissolved in 50% ethyl alcohol, fast green stain dissolved in absolute ethyl alcohol, clove oil, *Canada balsam*. Drying: carried in an oven adjusted at 60°C. Microscopical examination: by using Olympus CH2O microscope. The lens used was (×20, ×40), and photographing by using (LeitzDialux 20) microscope fitted with (Wild PMPS II) camera, using Kodak coloured films 36 ExP. 24 × 36 mm ISO 100/210.

Purity tests

Determination of total ash, acid insoluble ash, water soluble extractive, and determination of ethyl alcohol (70%) soluble extractive were carried out by following the methods described in (IHP, 2002). The determination of petroleum ether soluble extractive and determination of moisture content were carried out by using the methods described in Pulok (2002). The foreign organic matter determination was carried out by following the method described in (WHO, 1998).

Determination of heavy metals

Analysis was made on a known 0.5 g of the dried plant sample. Digestion was conducted using a microwave oven (milestone mps1200 mega). The conditions for wet ashing were carried as described by Shaole et al. (1997). Total content of cadmium and lead were determined in the digested solution using inductively coupled plasma (400) emission spectrometry (Perkin elemer emission spectrometer) according to Allen et al. (1997).

Determination of pesticide residues

Determination of pesticide residues was carried out by gas chromatographic multi residue quantitative determination of organohalogen, organonitrogen, organophosphrous and some pyrithroids pesticide residues, an official method for analysis (Association of Official Agricultural Chemists, 1995), adopted by Agricultural Research Center; Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Food, Egypt.

Determination of microbial contaminants

Pretreatment of the plant material: Ten grams of the grinded plant material was dissolved in 100 ml lactose broth enrichment medium, mixed well for homogenization and incubated at 37°C for 3 h so that *Esherichia coli* and *Salmonella typhimurium* can revive if present, to enable their detection (WHO, 1998).

Isolation and identification of *E. coli*: One ml of the homogenized material from the enriched plant material was transferred to a bottle containing 100 ml of MacConkey broth and incubated at 43 to 44°C for 20 h. A drop from the bottle was plated separately on MacConkey agar. Another drop was transferred to a tube of peptone water (for indole test). Plate and tube was incubated at 43 to 44°C for 20 h. Growth of red, generally non-mucoid colonies, sometimes surrounded by a reddish zone of precipitation, indicates the possible presence of *E. coli*, which may further be confirmed by indole reaction. The material passes the test if no such colonies are detected or if the confirmatory biochemical reaction is negative.

Isolation and identification of *Salmonella typhimurium*:

Primary test: The test was carried out by following the method described in WHO (1998). The appearing colonies should be compared with the description included in the same method.

Secondary test: The following biochemical reactions were performed for the plant material preparation: oxidase, urease, indole and Kligler iron agar (deep inoculation technique); all incubated at

Figure 1. Plant material of *Hibiscus sabdariffa* calyx.

incubated at 37°C for 24 h and confirmed by api 20E identification system (Eiman et al., 2008). The material being examined passes the test if colonies of the type described in the WHO (1998) do not appear in the primary test, or if the confirmatory biochemical reaction is negative.

HPLC determination of flavonoids in Hibiscus sabdariffa

One gram accurately weighed to prepare test solution of *Hibiscus* powder, 5 ml of methanol was added and 20 ml distilled water, the mixture was sonicated for 10 min and cooled. The amount transferred to 50 ml volumetric flask, 10 ml of dilute HCl acid was added and shaken well, completed to the volume using methanol and the solution was then filtered. 12 mg of quercetin standard accurately weighed to prepare standard solution, transferred quantitatively into 50 ml volumetric flask, dissolved and completed to volume with methanol. The test solution and quercetin standard sample were co-chromatographed on C_{18}-HPLC Column = Hypersil ODS C_{18}250×4.6 mm, HPLC (Hewlett Packard) series 1050 (Germany). A mixture of citric acid solution: acetonitrile: isopropyl alcohol (100:47:5) was used as mobile phase at flow rate 1.5 ml/min at room temperature. 20 µl from test and standard sample was injected separately into the HPLC system and the peaks monitored by UV absorbance at λ_{max} 370 nm (Mattila et al., 2000).

RESULTS AND DISCUSSION

Macroscopical characters *of Hibiscus sabdariffa* calyx

The macroscopical characters of *H. sabdariffa* calyxes are bright red calyxes 1.5 to 2 cm long (Figure 1). These morphological features are identical to WHO (1999) and Morton (1987).

Microscopical characters *of Hibiscus sabdariffa*

Transverse section showed elongated epidermal cells, vascular bundles and pigments cells (Figure 2) and the horizontal section showed clusters of calcium oxalate concentrated around the vessels in rows enclosed in parenchyma cells (Figure 3). These findings coincide with those stated in WHO (1999).

Purity tests of *Hibiscus sabdariffa*

Results show high numerical value obtained as water soluble extractive value (16.3), with considerable value for the 70% ethanol soluble extractive (21.4%), which indicates that the drug contains large amounts of somewhat polar constituents such as phenolic compounds, organic acids and minerals. This is in agreement with the conclusion obtained by German commission (1990). The total ash and acid insoluble ash values of *H. sabdarriffa* were calculated to be 11.7 and 3.8%, respectively. The values of ash and acid insoluble ash (not exceeding 2%) may not be constantly the same for the plant sample which indicate contamination with siliceous material (earth and sand). Also these differences may result from the change in climate, soil and age of the plant and/or change in care taken when preparing drugs (WHO, 1999, 2002; Pulok, 2002). The value of the total ash for Hibiscus has been set at 10% by Plotto (1999), which showed reasonable results when compared with the

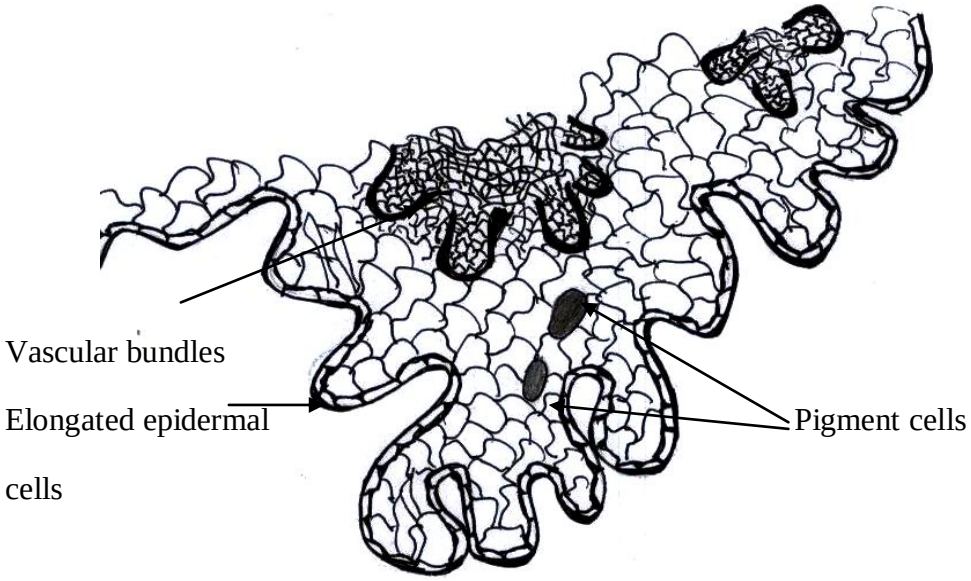

Figure 2. Transverse section of *Hibiscus sabdariffa* showing elongated epidermal cells, vascular bundles and pigments cells.

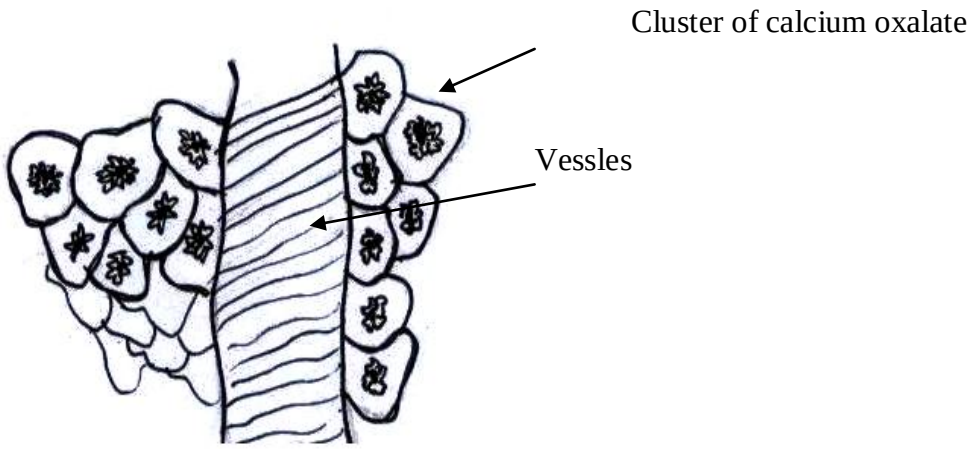

Figure 3. Horizontal section through the sepals of *Hibiscus sabdariffa*, showing clusters of calcium oxalate concentrated around the vessels in rows enclosed in parenchyma cells.

results obtained in this monograph. The suggested results by (Juliani et al., 2009) to obtain higher quality of *Hibiscus calyx*, a maximum of 7.5 and 1% are recommended for total and acid insoluble ashes, respectively. These results are not in agreement with the results obtained in this study. This may be attributed to variation in soil, good agricultural and collection practice (GACP). Value of moisture content was calculated to be 8.5%, as shown in Table 1. This value no doubt affects quality and safety of the medicinal plants as the higher moisture content leads to the deterioration of the drug. Most drugs may be stored safe if the moisture content is reduced to 6 percent or less (Pulok, 2002). For pesticide residues and as mentioned in Table 1, *Hibiscus* is not contaminated with profenofos, chlorpyrifos and malathion. Value of foreign materials was calculated to be 2%, as shown in Table 1. Low foreign materials and low microbial load reported by Juliani et al. (2009) showed important parameter in quality, and health is the production and assurance of clean and hygienic products

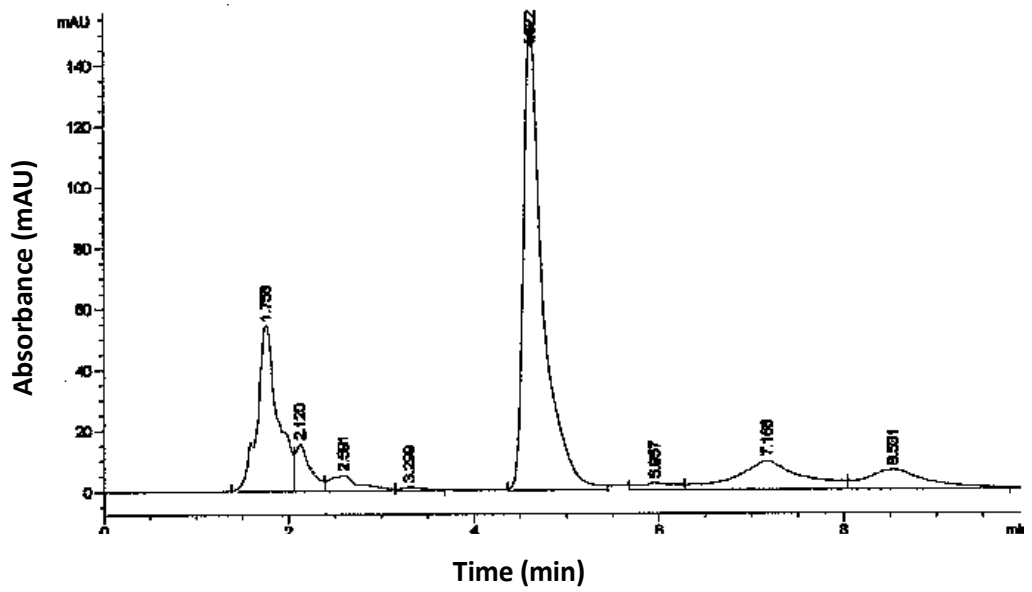

Figure 4. HPLC Chromatogram of quercetin in *Hibiscus sabdariffa* test solution.

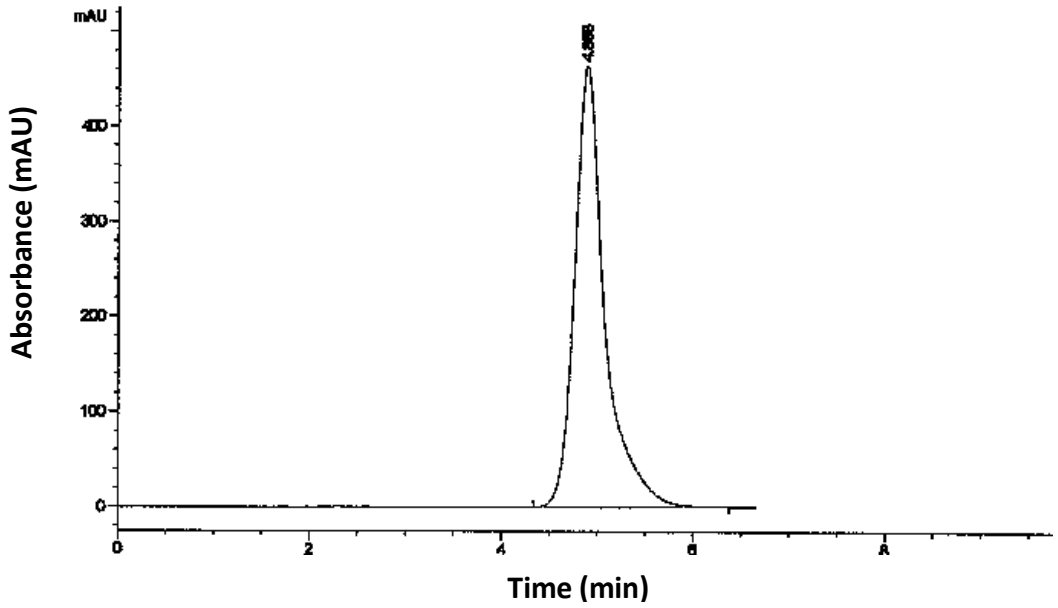

Figure 5. HPLC Chromatogram of standard quercetin solution.

that are safe to consume. The test sample was free from bacterial contamination with *Salmonella* and *E. coli* as mentioned in Table 1. The heavy metal lead in the plant sample tested was found to be below the permissible limit (10 mg/kg), while cadmium was not detected at all as mentioned in Table 1.

HPLC determination of flavonoids in *H. sabdariffa*

The HPLC analysis of *H. sabdariffa* revealed the presence of quercetin as a major phytoconstituent in a total content of 12.96% with a retention time 4.62 min (Figure 4) similar to the authentic reference sample (Figure 5). This

Table 1. Purity characteristics of *Hibiscus sabdariffa*.

Item	Numerical value
Acid insoluble ash	Not more than 3.8%
Water soluble extractive	Not less than 16.3%
70% ethanol soluble extractive	Not less than 21.4%
Petroleum ether soluble extractive	Not less than 0 .02%
Moisture contents	Not more than 8.5%
Foreign organic matter	Not more than 2%
Heavy metals	
Lead	0.0933 ppm
Cadmium	0.00 ppm
Pesticide residues	Not detected
Microbial contaminants	Free of *E. coli* and *S. typhimurium*

result was consistent to that reported by Salah et al. (2002).

Conclusion

It can be concluded that, obtained data of *H. sabdariffa*could be taken as guidelines in its quality assessment and will definitely contribute positively to establish standards for the quality assessment of herbal products in Sudan.

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REFERENCES

Alexander JD (1940). Plant Microtechniques. McGraw Hill, London.
Ali-Bradeldin H, Al-Wabel, N, Gerald B (2005). Phytochemical pharmacological and toxicological aspects of *Hibiscus sabdariffa*: A review. J. Phytother. Res. 19:369-375.
Allen IB, Sitonen PH, Thomposoy HC (1997). Methods for determination of arsenic, cadmium, copper, lead and tin in sucrose, corn syrups and high fructose corn syrups by inductively coupled plasma spectrometry. J. Agric. Food Chem. 45(1):162-165.
Amit J, Sunil C, Vimal K, Anupam P (2007). Phytosomes: A revolution in herbal drugs. Pharmaceut. Rev. pp. 11-13.
Association of Official Agricultural Chemists (1995). AOAC Official methods of analysis, 16th edition: 985-1022.
Azza A, Ferial M, Esmat A (2011). Physicochemical properties of natural pigments (anthocyanin) extracted from Roselle calyces (*Hibiscus sabdariffa).* J. Am. Sci. 7(7):445-456.
Eiman HA, Ashraf NA, Alamin MI, Hassan EK (2008). Microbial contaminants of selected Sudanese Medicinal plants and their compliance with quality control standards. Omdurman J. Pharmaceut. Sci. 1(4):397-561.
German Commission (1990). E. Monograph BANZ no 22a dated 1.2
Kunle O, Folashade E, Henry O (2012). Standardization of herbal medicines - A review. Int. J. Biodiv. Conserv. 4(3):101-112.
Juliani HR, Yudy Y, Dan S, Simon JE (2009). "Nutritional assessment of *Maringa* (*Moringa* spp.) From Ghana, Senegal and Zambia. African Natural Plant Products: New Discoveries and Challenges in Chem. Quality 469–484.
Indian Herbal Pharmacopoeia (IHP) (2002). Published by Indian Drug Manufacturers Association, Mumbai, Revised new edition.
Mattila P, Astola J, Kumpulainen J (2000). Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. J. Food Chem. 48:5834-5841.
Morton J (1987). Roselle. In: Fruits of warm climates. Handbook of energy crops. pp. 281-286.
Neeraj C, Bhupinder S (2011). An overview of advances in the standardization of herbal drugs. J. Pharm. Educ. Res. 2(2):55-75.
Patel PM, Patel NM, Goyal RK (2006). Quality control of herbal products. Indian Pharmacist 5(45):26-30.
Plotto A (1999). "Hibiscus: Post-Production Management for Improved Market Access for Herbs and Spices." Compendium on Post-Harvest Operations.
Pulok KM (2002). Quality control of herbal drugs, published by Business Horizons, first edition.
Salah AM, Gathumbi J, Vierling W (2002). Inhibition of intestinal motility by methanolic extracts of *Hibiscus sabdariffa* L. (Malvaceae) in rats. Phytother. Res. 16:283–285.
Shaole W, Xinbang F, Wittmeir A (1997). Microwave digestion of plant grain reference materials in nitric acid or mixture of nitric acid and hydrogen peroxide for the determination of multi-elements by inductively coupled plasma spectrometry. J. Analyt. Atomic Spectrometry 12(3):796-806.
World Health Organization (WHO) (1998). Quality control of medicinal plant materials, Geneva.
World Health Organization (WHO) (1999). Monographs on selected medicinal plants, volume 1, Geneva.
World Health Organization (WHO) (2002). Drug information, 14:4.
Yadav NP, Dixit VK (2008). Recent approaches in herbal drug standardization. Int. J. Integr. Biol. 2:195-203.

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Phytochemical and antimicrobial studies on extractives of *Calyptrochilum emarginatum* (SW) Schltr (Orchidaceae) growing in Nigeria

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Calyptrochilum emarginatum **is an epiphytic shrub belonging to the orchid family with numerous medicinal uses. Phytochemical investigation of the leaf extractives revealed the presence of tannins, flavonoids, carbohydrates, terpenes, sterols and saponins. Alkaloids and cardiac glycosides could not be detected. Antimicrobial studies of the extracts revealed that at a minimum inhibitory concentration (MIC) of 1.6 mg/ml, the hexane and methanol successive extracts exhibited bactericidal activities against *Staphylococcus aureus*. The straight run methanol extract and the successive ethyl acetate extract did not show any activity against all the microorganisms investigated namely, *S. aureus, Candida albicans, Klebsiella pneumonia, Escherichia coli* and Salmonella paratyphi at the same concentration.**

Key words: *Calyptrochilum emarginatum*, orchid, phytochemicals, antimicrobial, minimum inhibitory concentration (MIC).

INTRODUCTION

Calyptrochilum emarginatum (Afzel. ex Sw.) Schltr is an epiphytic shrub belonging to the orchid family. Orchid is an anglicized name given to every plant species belonging to the family Orchidaceae. Like other epiphytes, *C. emarginatum* grows on tree branches, unconnected to the ground and without being parasitic in any way. The leaves grow along a pendant stem up to 50 cm long with inflorescence appearing along the underside of the stem with 6 to 9 flowers. *C. emarginatum* is a shade-loving shrub with stems carrying distichous, ovate, leathery, unequally and obtusely bilobed (emarginated) apical leaves arising with an auxiliary. Flower inflorescence is characterized by strong scent, even nocturnal. The plant flowers approximately within 2 to 3 years. *C. emarginatum* can be found in Angola, Cameroon, Central African, Equatorial Guinea, Gabon, Ghana, Ivory Coast, Liberia, Nigeria, Sierra Leone and Zaire in tropical, evergreen and deciduous rainforest at elevation around sea level to 1000 meters (La Croix and La Croix, 1997).

There are only two recognized and acceptable species of *Calyptrochilum* and these are *C. chrystyanum* and *C. emarginatum.* Jayeola and Thorpe (2008) carried out electron micrograph of the genus *Calyptrochilum*. Kraenzl established taxonomical characteristics of the two species. *Chrystyanum* (Rchb.) Summerh was characterized by a network of horizontal grooves, dome shaped micro papillae and a mass of soft wax while the

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Emarginatum (Sw.) Schltr was distinguished by the presence of densely overlapping conical and globular type of micro papillae (Jayeola and Thorpe, 2008).

The medicinal orchids belong mainly to the genera: *Calanthe, Coelogyne, Cymbidium, Bulbophyllum, Cypipedium, Dendrobium, Ephemerantha, Eria, Galeola, Gastrodia, Gymnadenia, Habenaria, Ludisia, Luisia, Nevilia* and *Thunia* (Gutiérrez, 2010). Through the ages, several health-promoting benefits have been attributed to the use of orchid extracts such as anti-diuretics, anti-inflammatory, anti-carcinogenic, hypoglycemic activities, anti-microbial, anti-convulsive, neuroprotective and anti-viral activities (Gutierrez, 2010). For instance, the water soluble decoction of the whole plant of *Anoectochilus formosanus* was found to show potency for tumor inhibitory activities in experimental animals after subcutaneous transplantation of CT-26 murin colon cancer cells (Tseng et al., 2006). As a part of a screening study, methanol extract of the leaves of *Spiranthes mauritianum* (Orchidaceae) was found to possess anti-inflammatory activity and showed antibacterial activities against some Gram-positive organisms (Matu and Van Staden, 2003). In a study of herb extracts from Chinese medicinal plants, it was found that *Bletilla striata* (Orchidaceae) possessed antioxidant and anti-microbial properties (Luo et al., 2007). *Bletilla striata* tubers collected with a non-metal cutting tool, cleaned and dried were used to treat tuberculosis, hemoptysis, gastric and duodenal ulcers, as well as bleeding, and cracked skin on the feet and hands. Other uses in China, Mongolia, and Japan include the introduction of euphoria, purification of blood, strengthening and consolidation of lungs, as well as the treatment of pus, boils, abscesses, malignant swellings, ulcers, and breast cancer. Additional medical applications of the boiled or dried tubers of *B. striata* include treatment of flatulence, dispepsia, dysentery, fever, malignant ulcers, gastrointestinal disorders, hemorrhoids, anthrax, malaria, eye diseases, tinea, ringworm, tumors, and necrosis, silicosis, traumatic injuries, coughs, chest pain, tuberculosis, vomiting of blood, gastrorrhagia, enterorrhagia, internal bleeding, inflammation, and chopped skin. The powdered roots mixed with mineral oil have been used as an emollient for burns and skin diseases. Whole plant preparations are tonic and used as treatment against leucorrhea, hemoptysis and purulent coughs. Leaves collected in the autumn are reported to cure lung disease (Kong et al., 2003).

Two stilbenoids namely 3,3'-dihydroxy-2',6'-bis(p-hydroxybenzyl)-5-methoxybibenzyl and 3',5-dihydroxy-2-(p-hydroxybenzyl)-3-methoxybibenzyl were isolated from the methanolic extracts of tubers of *B. striata* which showed inhibitory effect of tubulin polymerization at IC_{50} of 10 µM. Also, 7,8-dihydro-5-hydroxy-12,13-methylene dioxy-11-methoxyldibenz[b*f*]oxepin, 7,8-dihydro-4-hydroxy-12,13-methylenedioxy-11-methoxyldibenz [b*f*]oxepin; and 7,8-dihydro-3-hydroxy-12, 13-methylenedioxy-11-methoxyldibenz [b*f*]oxepin, cumulatin, densiflorol A and plicatol B isolated from *Bulbophyllum kwangtungense* exhibited anti-tumor activities (Gutierrez et al., 2010).

The forkloric uses of *C. emarginatum* among the Takkad people of Southern Kaduna in Northern Nigeria have been reported to include treatment of infant convulsive fever (Mathias et al., 2007). In this work, we investigated the phytochemical and antimicrobial properties of the extractives of *C. emarginatum* found growing in Western Nigeria where people frequently use this plant in traditional medicine for the management of cough, tuberculosis and malaria.

MATERIALS AND METHODS

Chemicals and reagents

Reagents used were of Analar grade and unless otherwise stated were procured from Zayo-Sigma Abuja.

Plant

The plant material was collected in the month of June, 2012 in front of Conference Centre of the Obafemi Awolowo University, Ile-Ife, Osun State, South-West of Nigeria. The site of collection was characterized and geo-referenced N07 31.536, E004 31.536, with elevation 269 m, using a GARMIN GPS 60 Global Positioning System (GPS). The plant was identified and authenticated by Mr. Gabriel and Mr. Bennard at the herbarium of the Department of Botany, Obafemi Awolowo University, Ile-Ife, South-West of Nigeria.

Plant preparation and extraction

The fresh leaves were chopped into smaller bits and oven dried at a temperature of 40°C for a period of two weeks until constant weight. The dried plant sample was pulverized using mortar and pestle, and passed through sieve No. 22 of 710 µm nominal mesh aperture. Then 30 g of the powdered material was weighed and extracted with 300 ml of hexane in a stoppered container for 24 h at ambient temperature (28 to 30°C), with shaking during the first six hours at 120 rpm. The resultant mixture was vacuum filtered with Whatman No. 1 filter paper. The filtrate was concentrated to dryness using rotary evaporator at 40°C to yield the hexane extract of *C. emarginatum* (HECE). The air dried marc (29 g) was extracted successively with ethyl acetate and methanol for 24 h each, at ambient temperature (28 to 30°C). A separate straight run methanol extract was obtained using 30 g of the powdered sample and macerating with 300 ml of methanol for 24 h at ambient temperature (28 to 30°C), with shaking during the first six hours. The resultant mixture was vacuum filtered with Whatman No. 1 filter paper. The filtrate was concentrated to dryness using rotary evaporator at 40°C to yield *C. emarginatum* methanol extract straight-run (CEMEST). The dried successive ethyl acetate and methanol extracts of *C. emarginatum* were designated EECE and CEME, respectively. The yields of all the extractives were determined.

Phytochemical analyses

The phytochemical analyses of the powdered herb and extractives were performed using the methods described by Harborne (1998),

Evans (2002) and Sofowora (2008), with some modifications. The analyses were carried out in triplicate.

Test for alkaloids

One gram of the powdered herb was macerated with 10 ml of methanol at room temperature (28 to 30°C) for 3 h, with shaking at interval. The mixture was filtered with Whatman No. 1 filter paper. The filtrate was evaporated to dryness on a boiling water bath. Then the residue or 20 mg of each of the extractives were separately extracted with 10 ml of 1% HCl and filtered. The solution was divided equally into five test tubes, and two drops of the following reagents were added to the respective test tubes: Mayer's reagent (potassium mercuric iodide solution); Dragendorff's reagent (potassium bismuth iodide solution); Wagner's reagent (solution of iodine in potassium iodide); Hager's reagent (a saturated solution of picric acid); and 10% tannic acid solution. The formation of amorphous or crystalline precipitates or coloured precipitate in at least three or all of these tests indicate the presence of alkaloids.

Test for flavonoids

Lead acetate test for flavonoids

Two gram of the powdered herb or 10 mg of the extracts were wetted with acetone, and the acetone completely evaporated on a boiling water bath. The residue was extracted with 10 ml of warm distilled water and filtered. 3 ml of the filtrate was placed in a test tube and two drops of 10% (w/v) lead acetate solution added. The formation of a cream coloured precipitate indicates the presence of flavonoids (Evans, 2002).

Sodium hydroxide test for flavonoids

To 3 ml of the filtrate obtained earlier was added equal volume of 10% (w/v) sodium hydroxide solution. The formation of yellow coloured solution indicates the presence of flavonoids (Evans, 2002).

Test for terpenes and sterols

One gram of the powdered herb was extracted by maceration with 10 ml of chloroform. The extract was filtered and evaporated to dryness on water bath. The residue or 10 mg of extract was dissolved in 5 ml of anhydrous chloroform and filtered. The filtrate was divided into two equal portions and used for Lieberman-Burchard test for terpenes and Salkowski's test for sterols, according to (Sofowora, 2008) as indicated.

Lieberman-Burchard test for terpenes

To the first portion of the chloroform solution was added 1 ml of acetic anhydride and shaken. Then 1 ml of concentrated sulphuric acid was added down the wall of the test tube to form a layer underneath. The formation of a reddish-violet colour at the lower layer indicates the presence of terpenes (Sofowora, 2008).

Salkowski's test for sterols

Two mills of concentrated sulphuric acid was carefully added to the second portion of the chloroform solution, so that the sulphuric acid formed a lower layer. A reddish- brown colour at the interface indicates the presence of a steroidal ring.

Test for saponins

One-half gram of the powdered herb or 10 mg of the extract was added to 5 ml of 95% ethanol and boiled for 2 min. The mixture was filtered with Whatman No. 1 filter paper into a clean test tube. Then 10 ml of distilled water was added and shaken vigorously for about 30 s. The formation of a persisting honey comb indicates the presence of saponins (Evans, 2002).

Test for tannins

Ferric chloride test for tannins

10 ml of distilled water was added to 1 g of the powdered herb or 10 mg of the extract in a test tube and boiled for 3 min in a water bath. The mixture was allowed to cool and then filtered with Whatman No.1 filter paper. Then 1 ml of the filtrate was diluted with 4 ml of distilled water and two drops of 10% ferric chloride were added. Instant formation of blue-black or greenish coloured solution indicates the presence of tannins (Evans, 2002).

Test for anthraquinone derivatives (Borntrager's test)

About 0.5 g of the powdered herb or 10 mg of extract was placed in a dried test tube and 10 ml of chloroform added. The mixture was shaken for 5 min and filtered with Whatman No. 1 filter paper. To 3 ml of the filtrate equal volume of ammonia solution was added and shaken. Formation of a bright pink-red colour in the upper aqueous layer indicates the presence of free anthraquinones derivatives (Evans, 2002).

Test for carbohydrates (Molisch's test)

About 1 g of the powdered herb or 10 mg of the extract was boiled in 10 ml of distilled water on a water bath for 3 min. The mixture was filtered while hot. A few drops of Molisch's reagent was added to 2 ml of the cooled filtrate and shaken, and then a small quantity of concentrated sulphuric acid was added and allowed to form a lower layer. The formation of a purple ring at the interface indicated the presence of carbohydrates.

Keller-Kiliani's test for cardiac glycosides

About 0.5 g of the herb or 10 mg of the extract was placed in a test tube. Then 5 ml of water and 2 ml of glacial acetic acid containing one drop of 10% ferric chloride solution were added. The contents were thoroughly mixed and filtered. To 2 ml of the filtrate, 1 ml of concentrated sulfuric acid was added down the wall of the test tube to form a layer underneath. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides, indicative of cardiac glycosides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout the layer (Ayoola et al., 2008).

Antimicrobial activities

The four extracts were screened for antimicrobial activity against clinical isolates namely *Staphylococcus aureus, Candida albicans, Klebsiella pneumonia, Escherichia coli* and *Salmonella paratyphi*

Table 1. Colour and yields of dried extracts of *C. emarginatum*.

Extract	Colour	Yield (g)	Yield percentage (w/w)
HECE	Greenish	0.75	0.25
EECE	Greenish	0.51	0.17
CEME	Brown	0.46	0.15
CEMEST	Greenish brown	1.53	0.5

HECE = successive hexane extract of *C. emarginatum,* EECE = successive ethyl acetate extract, CEME = successive methanol extract, CEMEST = straight run methanol extract.

Table 2. Results of the phytochemical analyses of *Calyptrochilum emarginatum* herb and extracts.

Secondary metabolite	Herb	HECE	EECE	CEME	CEMEST
Alkaloids	-	-	-	-	-
Tannins	+++	-	-	+++	+++
Flavonoids	++	-	++	++	++
Terpenes	++	++	++	+	++
Steroids	++	+	++	+	++
Cardiac glycosides	-	-	-	-	-
Carbohydrates	++	-	-	-	-
Saponins	++	-	-	++	++
Anthraquinones	-	-	-	-	-

HECE = successive hexane extract of *C. emarginatum,* EECE = successive ethyl acetate extract of *C. emarginatum,* CEME = successive methanol extract of *C. emarginatum*, CEMEST = straight run methanol extract of *C. emarginatum,* + = slightly present, ++ = present, +++ = highly present, - = not detected.

obtained from the staff clinic of the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria, using agar dilution streak technique (Mitscher et al., 1972). The test organisms were prepared by sub-culturing in freshly prepared nutrient broth at 37°C for 3 h, having approximately 1.25×10^6 to 1.25×10^7 colony forming units (cfu). Then 64 mg each of hexane and ethyl acetate extracts were dissolved in 2 ml dimethyl sulphur oxide (DMSO) to give a concentration of 32 mg/ml. Similarly, 64 mg each of the methanol extracts were dissolved in 2 ml of water. 1 ml of the prepared extracts each was introduced into 19 ml of molten nutrient agar placed in water bath at 45°C. These were mixed properly and poured into sterile petri dishes to give a final concentration of 1.6 mg/ml. The dishes which were prepared in duplicates were then allowed to gel and thereafter, the test organisms were inoculated by streaking onto the nutrient agar using a wire loop meant to deliver 0.002 ml containing approximately 2.5×10^3 to 2.5×10^4 cfu.

The organisms were also streaked on dishes containing only agar (organism viability control), dishes containing nutrient agar and DMSO and dishes containing nutrient agar and water, which also served as controls. The petri dishes were incubated for 24 h at 37°C after which they were observed for microbial growth inhibition. Both water and DMSO showed no inhibiting effect on the organisms.

Plates where growth inhibition of the organisms was observed were further incubated for 48 h and then a little of the inhibited organisms were sub-cultured in a freshly prepared nutrient broth and then incubated for 24 h at 37°C. Observation of clear broth indicates bactericidal activity while turbidity of the broth indicates bacteriostatic activity.

RESULTS

The phytochemical and antimicrobial properties of the extractives were determined. As shown in Table 1, the combined extractive value of HECE, EECE, and CEME obtained from successive extraction was higher than that from the straight run methanol extraction CEMEST. However, considering the individual extractive values, CEMEST had the highest extractive value. As shown in Table 2, the methanol extractives CEME and CEMEST contained majority of the phytochemicals detected namely tannins, flavonoids, terpenes, sterols and saponins. Antimicrobial studies of the extracts revealed that at a minimum inhibitory concentration (MIC) of 1.6 mg/ml, the hexane and methanol successive extracts exhibited bactericidal activities against *S. aureus*. The straight run methanol extract and the successive ethyl acetate extract did not show any activity against all the microorganisms investigated namely, *S. aureus, C. albicans, K. pneumonia, E. coli* and *S. paratyphi* at 1.6 mg/ml antimicrobial activities at the same concentration (Table 3).

Table 3. Antimicrobial activity of *C. emarginatum* extracts.

Extract	Concentration (mg/ml)	Microorganism	Biocidal activity	Biostatic activity
CEMEST	1.6	Sa	-	-
		Ca	-	-
		Kp	-	-
		Ec	-	-
		Sp	-	-
HECE	1.6	Sa	+	-
		Ca	-	-
		Kp	-	-
		Ec	-	-
		Sp	-	-
EECE	1.6	Sa	-	-
		Ca	-	-
		Kp	-	-
		Ec	-	-
		Sp	-	-
CEME	1.6	Sa	+	-
		Ca	-	-
		Kp	-	-
		Ec	-	-
		Sp	-	-

HECE = successive hexane extract of *C. emarginatum;* EECE = successive ethyl acetate extract of *C. emarginatum;* CEME = successive methanol extract of *C. emarginatum*; CEMEST = straight run methanol extract of *C. emarginatum;* Sa = *Staphylococcus aureus*; Kp = *Klebsiella pneumonia*; Sp = *Salmonella paratyphi*; Ca = *Candida albicans*; Ec = *Escherichia coli;* + = inhibition; - = no inhibition.

DISCUSSION

Phytochemical analyses

The colour and yields of the extracts studied are shown in Table 1. HECE and EECE were green in colour and had yields of 0.25 and 0.17% w/w, respectively. The successive methanol extract CEME was brown and the yield was 0.15%. On the other hand, CEMEST was greenish brown and the yield was 0.5% w/w. The results of the phytochemical tests of the herb and extracts of *C. emarginatum* presented in Table 2 showed that six major classes of secondary metabolites were detected namely, terpenes, sterols, saponins, tannins, flavonoids and carbohydrates. HECE contained terpenes and sterols. EECE contained terpenes, sterols and flavonoids. CEME and CEMEST contained terpenes, sterols, flavonoids, saponins and tannins. The methanol extracts CEME and CEMEST contained the majority of the secondary metabolites detected in which polyphenols are predominant. The phytochemical constituents of *C. emarginatum* from Northern Nigeria included flavonoids, saponins, steroids, terpenoids and tannins (Mathias et al., 2007), which is consistent with findings reported herein.

Antimicrobial studies

The four extracts HECE, EECE, CEME and CEMEST were screened against five microorganisms at 1.6 mg/ml concentration. As shown in Table 3, antimicrobial studies of the four extracts revealed that at 1.6 mg/ml concentration, the hexane extract (HECE) containing terpenes and steroidal compounds, and the methanol successive extracts (CEME) rich in saponins and polyphenols were active against *S. aureus* at 1.6 mg/ml. The straight run methanol extract (CEMEST) and the ethyl acetate successive extract (EECE) did not show any activity against all the microorganisms investigated namely, *S. aureus, C. albicans, K. pneumonia, E. coli* and *S. paratyphi*, at the same concentration of 1.6 mg/ml. HECE and the CEME inhibited the growth of *S. aureus*. On further observation, bactericidal activity was established.

Antimicrobial activity observed at the concentration of 1.6 mg/ml by any agent is indicative of potent activity (Mitscher et al., 1972). The antimicrobial activity result from this study supports the folkloric use of *C. emarginatum* for the treatment of respiratory tract and opportunistic infections.

Conclusion

C. emarginatum is rich in phytochemicals, some of which may be attributed to its ethnomedicinal uses for management of coughs, tuberculosis, and as antimalarial. The presence of some secondary metabolites like flavonoids, saponins, tannins, phenols, terpenes and sterols, all of which have been reported to exhibit physiological activities in man, animals and microorganisms, suggests that the plant may be used as a potent vegetable drug. Some phytochemicals are used in the pharmaceutical industry for the production of various drugs. Examples include the anticancer taxol as paclitaxel, artemisinin and its derivatives artesunate and artemether as antimalarials (Evans, 2002).

Flavonoids and saponins have been reported to possess antioxidants, hepatoprotective and anti-inflammatory activities, and are used as antimicrobial, anticancer and antiallergic remedies. Some tannins had been reported as anti-viral and anti-tumor agents as well as diuretics. Terpenes like the mono-, sesqui- and triterpenes, and sterols had been reported to exhibit various biological activities in animals and micro-organisms some of which include anti-inflammatory, anti-microbial and hormonal activities. Some steroidal compounds have been reported to exhibit anti-diabetic properties (Evans, 2002). The extracts from *C. emarginatum* namely HECE and CEME exhibited bactericidal activity against *S. aureus*, which is responsible for some respiratory tract and opportunistic infections. This finding corroborates the health-promoting benefits that have been attributed to the folkloric uses of *C. emarginatum*.

REFERENCES

Ayoola GA Coker HAB, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, Atangbayila TO (2008). Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in South-western Nigeria. Trop. J. Pharm. Res. 7 (3):1019-1024.

Evans WC (2002). Trease and Evans Pharmacognosy, 15th edition. W.B. Sanders London. pp. 214-393, 419.

Gutiérrez RMP (2010). Orchids: A review of uses in traditional medicine, its phytochemistry and pharmacology. J. Med. Plants Res. 4(8):592-638.

Harborne JB (1998). Phytochemical methods, a guide to modern technique of plant analysis. 3rd Edition Chapman and Hall. New York, pp. 1-198.

Jayeola AA, Thorpe JR (2008). A scanning electron microscope study of the genus Calyptrochilum Kraenzl. (Orchidaceae) in West Africa. Feddes Repertorium 111(5-6):315-320.

Kong JM, Goh NK, Chia LS, Chia TF (2003). Recent advances in traditional plant drugs and orchids. Acta Pharmacol. Sin. 24: 7–21.

La Croix E, La Croix IF (1997). African Orchids in the Wild and in Cultivation. Timber Press, Portland, Oregon. pp. 43-45.

Luo H, Lin S, Ren F, Wu L, Chen L, Sun Y (2007). Antioxidant and antimicrobial capacity of Chinese medicinal herb extracts in raw sheep meat. J. Food Prot. 70:1440-1445.

Mathias SN, Ilyas I, Musa KY (2007). Phytochemical constituents of some medicinal plants used amongst the Takkad people of Southern Kaduna-Nigeria. Chem. Class J. 4:70-75.

Matu EN, Van Staden J (2003). Antibacterial and anti-inflammatory activities of some plants used for medicinal purposes in Kenya. J. Ethnopharmacol. 87:35-41.

Mitscher LA, Leu RP, Bathala MS,Wu WN, Beal JL, White R (1972). Anti-microbial agents from higher plants. I. Introduction, rationale, and methodology. J. Nat. Prod. (Lloydia) 35:157-166.

Sofowora A (2008). Medicinal Plants and Traditional Medicine in Africa, Third edition. Published by Spectrum Books Limited, Ibadan, Nigeria. pp. 199-202.

Tseng CC, Shang HF, Wang LF, Su B, Hsu CC, Kao HY, Cheng KT (2006). Antitumor and immunostimulating effects of *Anoectochilus formosanus* Hayata. Phytomedicine 13:366-370.

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Full Length Research Paper

Seed treatments to break seed dormancy and Standardization of viability test procedure in *Abrus precatorious*

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Abrus precatorius **is an indigenous medicinal plant belongs to Fabaceae family and grow as wild vine in tropical and subtropical climate conditions. Seeds of this species posses seed dormancy and restricts germination to overcome unfavorable environmental conditions. This dormancy need to be removed to enhance germination under favourable condition of plant growth. Hence, different dormancy breaking treatments were imposed on freshly harvested seeds to improve germination. Treatments includes physical and physiological methods like soaking in water (24 h), conc. H_2SO_4 (2 min), KNO_3 (2%) (24 h), GA_3 100 ppm (24 h), Kinetin 100 ppm (24 h) and mechanically damaging the seed coat. The experimental results revealed that *A. precatorius* posses seed dormancy, mainly due to leathery testa leading to impermeability for water and oxygen so called hard seeds. Among treatments, damaging the seed coat (Nicking) enhanced germination from 32 to 84%, followed by seeds soaked in gibberlic acid (100 pm) for 24 h (78 %). In nature, dormancy was gradually reduced and found no dormancy behavior after seven months of harvest. For quick viability test, seed coat must be mechanically damaged before preconditioning of seeds for better results. Also, seeds soaked in Tz solution of 1.0 (%) for 6 h or 0.1% for 12 h helps for clear distinguishing of viable and non viable seeds in abrus.**

Key words: Abrus, medicinal plant, seed dormancy, seed viability, seed enhancement, germination.

INTRODUCTION

Seed dormancy is the resting period of seed after physiological maturity and also an adoption mechanism to overcome stress conditions. Seeds germinate when it come in contact with moisture at optimum temperature in the presence of oxygen. There are exceptional species, which does not germinate even under all favourable conditions, and this is because of the dormancy prevailing in the seeds. Majority of the medicinal plants are non domesticated, and they grow as wild plants. The wild nature has made the seeds dormant for their survival under unfavourbale conditions also this mechanism helps for their dispersal and perpetuations. Problem arises when these types of dormant species were adopted for commercial cultivation and this need to be addressed to improve the plant stand and yield.

Abrus precatorius is an indigenous medicinal plant which thrives well in tropical and subtropical climate conditions as a wild vine. It belongs to family Fabaceae and

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is a highly cross pollinated plant, and pollinating agents are mainly ants, bees and bugs (Ikechukwu et al., 2007). Leaves and roots of *Abrus* contain glycyrrhizin, the principal component of licorice. These tissues prepared in various ways are used to treat coughs and a number of other ailments (Parrotta, 2001). Its leaves are consumed as a vegetable and also used to prepare liquorice (Choi et al*.,* 1989) in Central and East Africa and also used against mouth boils (chew and spit), and seed are burnt to apply on wounds (Bhagya and Sridhar, 2009). Children have been reported to suck nectar from the flowers as a snack (Jakinovich et al., 1990). Whereas, seeds are poisonous when chewed by humans and animals; however, seeds are used to simulate eyes for sculpting in Southeastern Nigeria.

Further, seeds are beautiful and uniform in size and are red colour with black spot. Because of this attractive colour birds pick them through curiosity or by being momentarily deceived into thinking that they are edible and helps in seed dispersal to short distances (Galetti, 2002). Fresh seeds are poor germinators which may be because of hard seed coat, leading to dormancy (Plate 1). The presence of hard seed coat may be impermeability to oxygen (Crocker, 1916) or mechanically resistant or inhibitors present in the seed coat or combination of these factors lead to no germination of seeds. Germination has to be improved by removing dormancy and this will helps to enhance the germ plasm under cultivation.

Livingness of any of the seed can be determined by germination and quick viability test/Tz salt test. In germination test, the dormant seeds are wrongly interpreted as ungerminated poor quality seeds or dead seeds. This disadvantage can be overcome by the Tz test by evaluating its viability. TZ test gives an early and quick snapshot of seed viability even though seeds do not germinate due to the presence of dormancy. The main principle involved here is the conversion of colourless 2,3,5-triphenyl tetrazolium chloride into stable red colour compound called 2,3,5- triphenyl farmazon, in the presence of dehydrogenase enzymes which are an indication of livingness of cells. The preconditioning of the seeds is done to activate these dehydrogenase enzymes by imbibitions process. The concentration of the Tz solution and duration of exposure influence the conversion of Tz to farmazon. Hence, there is a need to standardize the preconditioning technique of the seeds, concentration of the solution and exposure period to interpret the viability test accurately (Plate 2).

MATERIALS AND METHODS

Fresh seeds of Abrus were collected from Northern dry zone of Karnataka, India (16° 12' (N), 75° 45' (E), 532 m) during Rabi season. Seeds were used for the study after drying seeds to 9% moisture content. 400 seeds per treatment were treated with different dormancy, breaking treatments in four replications. Treatments were imposed by soaking the seeds with solutions of T_1: plain water (24 h) T_2: H_2SO_4 (5 min) T_3: KNO_3 (2%) (24 h); T_4: GA_3 100 ppm (24 h); T_5: Kinetin 100 ppm (24 h); T_6: Nicking (damaging the seed coat away from embryo); T_7: Control (untreated). Concentration of the hormones was decided based on results obtained in previous studies of dormancy. The treated seeds were air dried under shade and then tested for its germination and vigour inbetween paper method according to International Seed Testing Association (ISTA) (Annon, 1996). The germination was evaluated on the 12th day after incubation in seed growth chamber at 28°C. The hard seeds were determined based on the seeds remaining fresh, unimbibed and hard at the end of the germination test (Annon, 1996). Natural breakdown of seed dormancy was studied by storing freshly harvested seeds containing 9% moisture in polythene (200 guage) bags under natural storage conditions. Further, stored seed was evaluated for its germination (%) and vigour at monthly intervals till seed lot recorded maximum germination (Plate 3).

Viability test

Standardization of the viability test procedure was conducted to optimize the concentration of Tz solution and period of soaking. Seeds were punctured (nicked) before soaking in water for 12 h as preconditioning treatment. At the end of the soaking period, hard seed coat became soft and was removed to expose the cotyledon. Naked cotyledon was transferred to the Tz solution of two concentration (C_1: 0.1 and C_2:0.5%) and soaked for different durations (D_1: 3 h D_2: 6 D_3: 12 and D_4: 24 h). The treatment was imposed at 30°C as enzymes are more active at this temperature. By the end of the treatment period, seeds were washed with water and evaluated by visual observation by keeping them under a dissection microscope. Cotyledon staining pattern and intensity building was observed at predetermined duration of soaking. Dissecting microscope visualized the pattern of staining and also the intensity of the colour clearly. The data obtained was analyzed for analysis of variance (ANOVA) and the means were statistically grouped by Tukey's test (Panse and Sukhatme, 1978).

RESULTS

Freshly harvested seeds showed very poor germination before any seed treatments, whereas different dormancy breaking seed treatments recorded positive response on germination because of seed treatment germination percent has increased, and decrease in hard seed percent was observed (Table 1). Germination has increased significantly from 32 to 84% after damaging the seed coat (nicking), resulting in 52% enhanced germination over control. Similarly, seeds treated with the gibberlic acid (100 ppm) for 24 h recorded germination of 78 and 15% hard seeds. Acid scarification for 2 min has also increased the germination up to 59% which was on par with 2% KNO_3 (57%) and kinetin 100 ppm (54%), whereas, the percent hard seed was 24% when scarified with acid. However, the increased duration of acid treatment resulted in an increased number of abnormal and dead seeds. Soaking of seeds in plain water for 24 h

Table 1. Influence of different dormancy breaking seed treatments on germination and seedling vigour in *Abrus precatorius.*

Treatment	Hard seed (%)	Germination (%)	Seedling length (cm)	Seedling vigour index
T_1: water (24 h)	35	55	12.33	678
T_2: H_2SO_4(2 min)	24	59	10.46	617
T_3: KNO_3(2%) (24 h)	32	57	13.16	750
T_4: GA_3 100 ppm (24 h)	15	78	12.46	971
T_5: Kinetin 100 ppm (24 h)	31	54	11.43	617
T_6: Puncturing seed coat/nicking	4	84	28.43	2388
T_7: Control	51	32	8.3	265
CD (0.01)	3.201	3.130	3.172	124
m±SE	1.523	1.662	1.702	1.576
CV (%)	2.95	2.62	9.46	4.53

also improved the germination significantly (55%) and reduced hard seeds (35%) over control.

Seed treatments imposed to break the seed dormancy have significantly increased the seedling length. Seeds after nicking have produced seedling of length 28.43 cm which was higher among the treatments. Short seedling was observed in untreated control (8.30 cm) followed by acid treatment (10.46 cm.). Seedlings of water soaked (12.33 cm), and growth hormones (12.46 and 11.43 cm in GA3 and kinetin, respectively) recorded significantly longer seedlings, and are on par with each other. Seed coat nicked away from embryo recorded significantly higher seedling vigour index (2,388) followed by seeds soaked in gibberlic acid (971). Poor seedling vigour was observed in untreated controlled seeds (266) followed by acid treatment (617). The other growth hormones and water soaking treatment also significantly increased the seedling vigour over control and are on par with each other.

Natural dissipation of dormancy was studied by storing the freshly harvested seeds, and initial germination was 32% without any seed treatment. Seeds were tested for its quality in monthly intervals (Figure 2). As the storage period progressed there were decreased number hard seeds and increased germination. The germination percent increased gradually from 32 to 86% at the end of 210 days of storage, whereas hard seeds were converted to normal seeds gradually during storage of 210 days. From this study, it was observed that *Aburs* seeds lost its dormancy naturally after seven months of harvest.

Seed viability test was conducted to standardize the concentration of Tz solution and exposure period for clear distinguishing of viable and non viable seeds. Seed coat need to be punctured to facilitate the easy imbibition of water and activation of enzymes. Seed coat removed cotyledons were used soaked in different concentration of Tz solution for different periods. Irrespective of the concentration of Tz solution, staining of seeds started only after 3 h of soaking period. Seeds soaked in 0.1% Tz solution took complete stain after 12 h of soaking period. Whereas, seeds soaked in 1.0% took only 6 h for complete staining. Higher concentrated solution (1.0%) stained faster than the lower concentration (0.1%). At the end of the test, Tz solution of 1.0% recorded 86% of seed staining after 6 h of soaking period, which remained constant even after 24 h of soaking period. Whereas, lower concentration of 0.1% took 12 h to stain 86% of seeds tested. At the end of 24 h of soaking, seeds stained deeply in both the concentrations which made difficult in the analysis of the results (Table 2).

DISCUSSION

Seeds of Fabaceae family exhibit dormancy because of hard testa impermeable to water and gases (Shaik et al., 2008; Ali et al., 2011). *A. precatorius* belongs to Fabaceae family and also shows hard seeds because of leathery seed coat which hinders the absorption of water and exchange of gas (Russi et al., 1992; Lopez et al., 1999). Abrin and agglutinin-I from the seed coat are type II ribosome-inactivating proteins that inhibit protein synthesis in eukaryotic cells inhibiting imbibitions process during germination (Bagaria et al., 2006). Hard seed formation in *Abrus* as a wild climber will improve its survival in soil and allows germination only when it comes across favourable conditions and confirms its existence in nature so as to avoid extinction. Seeds produced in Northern dry zone of Karnataka (tropical climate) have re-corded 51% hard seeds may be because of dry weather prevailing during the seed maturity stages as it also influence the percent of hard seeds formed (Quinlivan and Millington, 1962).

Seed dormancy due to hard seeds is removed by puncturing the seed coat, and the germination increased to 84% (Figure 1). The damage in the leathery testa has

Table 2. Seed viability staining as influenced by concentration and duration of soaking in TZ salt solution in *Abrus precatorius.*

Duration of soaking (h)	Concentration of Tz salt solution (%)		Mean
	0.1 %	1.0 %	
3	33	65.2	49.1
6	70.6	86	77.3
12	86	88	84.7
24	88.2	88	88.1
Mean	69.45	81.3	
CD (0.01)	**Concentration**	**Duration**	**Interaction**
	1.584	2.239	3.169
m±SE	3.283	-	-
Cv %	2.46	-	-

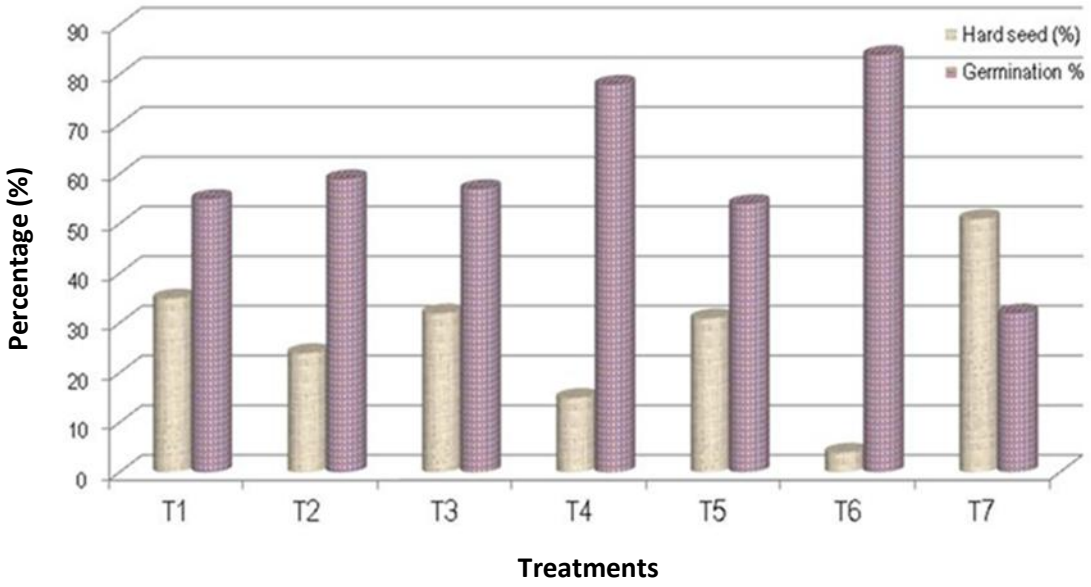

Figure 1. Influence of seed treatments on germination and hard seeds in *Abrus precatorius.* T_1: water (24 h); T_2: kinetin 100 ppm (24 h); T_3: KNO3 (2%) (24 h); T_4: GA3 100 ppm (24 h); T_5: kinetin 100 ppm (24 h); T_6: Nicking; T_7: control.

allowed the seeds for easy imbibition of water and exchange of gases and softening of seed coat. Further, softened seed coat has allowed the growing tips to emerge out from cotyledons by pushing seed coat apart. Imbibition process initiates the physiological process of germination and results in growth of plumule and radical. Imbibition as the first phase of germination was encouraged after seed treatments and hence seeds after water soaking have increased the germination over control. This attributed to activation of enzymes, swelling and softening of seed coat (Mudasir et al., 2012) and also leaching of germination inhibition chemicals presented in the testa (Tambat et al., 2006).

Similarly, seed treated with gibberlic acid has also increased the germination and decreased the number of hard seeds, and this is due to the activation of enzymes required for the energy generation leading to growth of the embryo. These growing tips were vigourous and hard enough to break open the seed coat and emerged as a seedling. This was also reported by Birgit et al. (2005) and proved that growth hormones release enzymes that break down carbohydrates, proteins and fats, which in

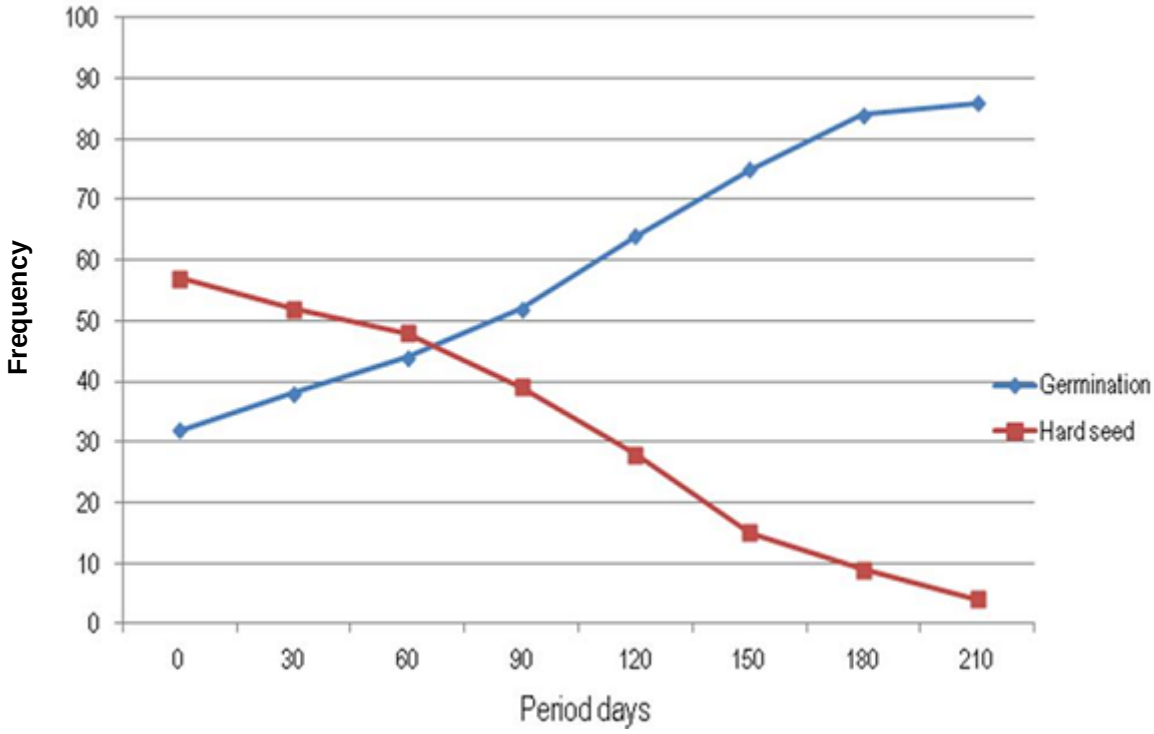

Figure 2. Germination and hard seeds under natural conditions during storage in *Abrus precatorius*.

turn release free sugars and also counteracts with inhibitors. Acid treatment is successful in reducing the hard seeds and increases the germination over control, since the acid had similar effect as that of natural degradation of the testa by microbial action in the soil (Van Staden et al., 1994).

Successful germination occurs when favourable conditions prevailed in the seed micro climate coupled with no growth inhibitors. Other seed treatments also made a positive impact on dormancy release and hence, these species are known to contain certain inhibitory compounds in seed coat and/or endosperm, which affects the germination process; external supply of growth hormones (GA_3, kinetin, KNO_3) neutralizes such effect and facilitates germination (Evenari, 1949; Mary, 1972).

Seedling length at the end of the germination test showed significant variation among the treatments. Seeds after nicking showed higher seedling length (28.43 cm) when compared to all other treatments, seedlings showed vigours growth from the third day of the test (Plate 4). The damaged seed coat gave the way for better absorption of water, better exchange of gasses resulted in rapid growth of seedlings compared to other treatments. Seeds soaked in water, and growth hormones also noticed better seedling length than control. In untreated control, the seedling length was short (8.3 cm), this may be due to the slow rate of water absorption which took more time for the activation of enzymes, and also the energy produced during the germination process has been utilized for break opening the leathery seed coat. Growth of the seedlings, slow, is due to the poor vigour of the dormant seeds (Mohamed et al., 1994). Seedling vigour index is also maximum in seed coat punctured seeds followed by seeds soaking in gibberlic acid. Increased germination and seedling length has contributed to the significant higher seedling vigour index, whereas, the vigour was also very poor in untreated controlled seeds.

Seed hardness of *Abrus* was naturally removed after 210 days of harvest. As the seed underwent ageing, the percentage of hard seeds decreased gradually (Figure 2). In nature, the biological cycle/rhythm will control the seed germination in order to ensure its survival in nature. Naturally, the dormancy of the seeds declines under natural conditions with time due to natural factors like disintegration of seed coat, leaching of germination inhibitors from the seed coat and microbial activities on the seed coat (Tran and Cavanagh, 1984; Van Staden et al., 1994; Mohamed et al., 1994), ultimately ensuring its survival in nature.

Concentration of the Tz solution and duration of soaking was standardized for quick viability test to know the livingness of seed. Concentration and the duration of soaking have positive relation on the seed staining pattern.

Plate 1. Germination of freshly harvested seeds before seed treatment in *Abrus precatorious.*

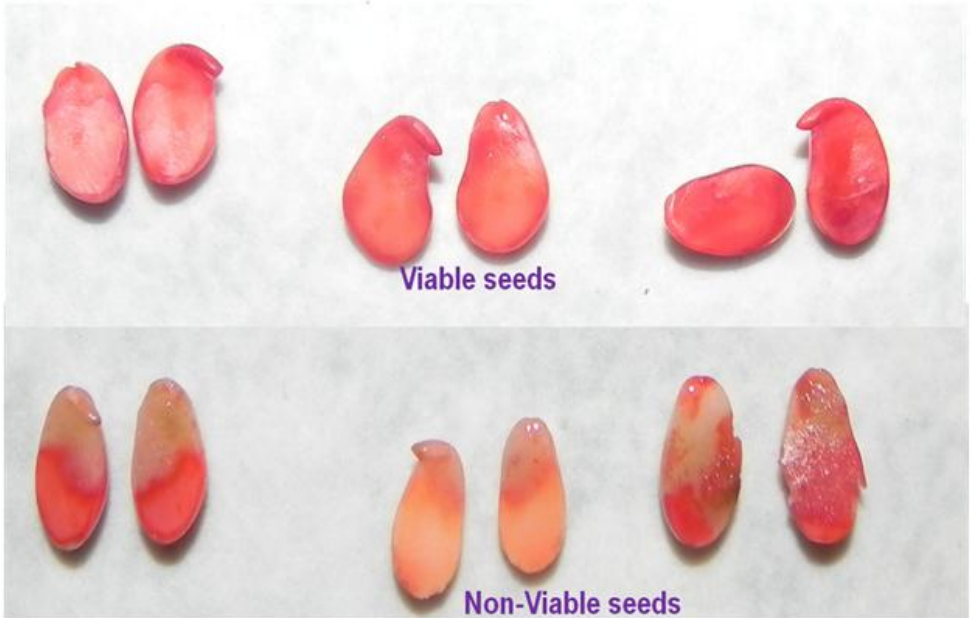

Plate 2. Viable and no viable abrus seeds after treating with 0.5% Tetrazolium chloride solution.

the early impact on the staining process than lower concentration. Availability of higher Tz has allowed the enzymes for conversion of Tz into farmozon in the presence of hydrogense enzymes of living seed cells. Similarly, period of soaking also influenced the staining process and observed that increase in soaking period resulted in the uniform staining of the seeds and made easy for evaluation of the viability. Also puncturing of seed coat helps easy absorption of water and quick enzyme activation. So punctured seeds soaked in 1.0%

Plate 3. Seed germination of abrus after GA_3 treatment.

Plate 4. Seed germination of abrus after nicking/puncturing of seed coat.

for 6 h showed clear staining and distinguishable pattern to identify viable and non viable seeds in *Abrus*.

Conclusion

Seed coat dormancy of *Abrus* can be overcome by making damage to the seed coat away from the embryo and it will enhance the germination. This method of dormancy breaking treatment need to be applied during seed germination test of *Abrus* also. Similarly, by soaking seeds in gibberlic acid (100 ppm) for 24 h also enhances the germination percent. Naturally, the seeds show dormancy for 210 days after which no dormancy is prevailing and ensures maximum germination. In nature, the release of dormancy is a gradual process, and complete removal of dormancy is only after seven months of seed ageing. And effective seed viability test can be conducted by soaking preconditioned puntured seeds in 1.0% Tz solution for 6 h or 0.1% Tz solution for 12 h.

REFERENCES

Ali HH, Tanveer A, Nadeem MA, Asghar HN (2011). Methods to break seed dormancy of *Rhynchosia capitata*, A summer annual weed. C. J. Agric. Res. 71(3):483-487.

Annon (1996). International rules for seed testing. Seed Sci. Technol. 24:1-228.

Bagaria A, Surendranath K, Ramagopal UA, Ramakumar S, Karande AA (2006). Structure-Function Analysis and Insights into the Reduced Toxicity of *Abrus precatorius* Agglutinin I in Relation to Abrin. J. Biol. Chem. 281:34465-34474.

Bhagya B, Sridhar KR (2009). Ethnobiology of coastal sand dunes legumes of South-west Coast of India. India J. Tradit. Knowl. 8(4):611-620.

Birgit K, Cohn MA, Leubner-Metzger G (2005). Plant hormones interaction during dormancy release and germination. Seed Sci. Res. 15(4):281-307.

Choi YH, Hussain RA, Pezzuto JM, Kinghorn AD, Morton JF (1989). Abrusosides A-D, four novel sweet-tasting triterpene glycosides from the leaves of *Abrus precatorius.* J. Nat. Prod. 52:1118–1127.

Evenari M (1949). Germination Inhibitors. Bot. Rev. 15:153-194.

Galetti M (2002). Seed dispersal of mimetic seeds: parasitism, mutualism, aposematism or exaptation? *In* Seed dispersal and frugivory: ecology, evolution and conservation (D. Levey, W.R. Silva & M. Galetti, eds.). CABI Publishing, Oxon. pp. 177-192.

Ikechukwu OA, Gordian CO (1992). Breeding system and pollination ecology of two *Abrus* species (Fabaceae) from Tropical West Africa. Acta Bot. Croat. 66(2):205–216.

Jakinovich W, Moon C, Choi YH, Kinghorn AD (1990). Evaluation of plant extracts for sweetness using the Mongolian gerbil. J. Nat. Prod. 53:190–195.

Lopez J, Devesa JA, Ruiz T, Ortega-Olivencia A (1999). Seed germination in *Genisteae* (Fabaceae) from South-West Spain. Phyton (Horn, Austria) 39(1):107- 129,

Mary RC (1972). Tannins as gibberellins antagonists. Plant Phy. 49:323–330.

Mohamed YY, Barringer SA, Costanza S (1994). The role of seed coats in seed viability. Bot. Rev. 60(4):426-439.

Mudasir Q, Fatima K, Haukat SK (2012). Effect of pre-treatment on the germination of *Abrus precatorius* Linn. by soaking in water. Indian J. Appl. Pure Biol. 27(1):105-108.

Panse VG, Sukhatme PV (1978). "Statistical Methods for Agricultural Workers" ICAR, New Delhi, pp. 1-22.

Parrotta JA (2001). Healing plants of Peninsular India. CAB International, Wallingford, UK. P 944.

Quinlivan BJ, Millington AJ (1962). The effect of a Mediterranean summer environment on the permeability of hard seeds of subterranean clover. Aust. J. Agric. Res. 13(3):377-387.

Russi L, Cocks PS, Roberts EH (1992). Hard-seededness and seed bank dynamics of six pasture legumes. Seed Sci. Res. 2(4):231-241.

Shaik S, Dewir YH, Singh N, Nicholas A (2008). Influences of pre-sowing seed treatments on germination of the cancer bush (*Sutherlandia frutescens*), a reputed medicinal plant in arid environments. Seed Sci. Technol. 36(3):795-801.

Tambat B, Vishwanath K, Chaithra GN, Hareesh TS (2006). Improved germination of *Gymnacranthera canarica* Warb. An endangered, endemic tree species of *Myristica* swamps, Western Ghats, India. Seed Sci. Technol. 34:625-630.

Tran VN, Cavanagh AK (1984). Structural aspects of dormancy. In: Murray DR (Ed.), Seed Physiology. - Academic Press, Sydney. 2:1-44.

Van Staden J, Kelly KM, Bell WE (1994). The role of natural agents in the removal of coat-imposed dormancy in *Dichrostachys cinerea* (L.). Plant Growth Regul. 14(1):51-59.

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