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Full Length Research Paper

Role of leucine rich repeat and Ig domain containing 1 (LINGO1) in demyelinating and neurological diseases and investigation of conserved residues by docking analysis for novel therapeutic options

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A demyelinating sickness is any ailment of the nervous system in which the myelin casing of neurons is injured. This harm weakens the transmission of signals in the pretentious nerves. Demyelinating diseases, like multiple sclerosis (MS) and Charcot-Marie-Tooth (CMT) disease, are categorized on the basis of the scratch of the myelin covering around neurons, because of swelling and gliosis in the central nervous system (CNS) and peripheral nervous system (PNS), respectively. In this current research, an amalgam approach of comparative modeling and molecular docking pursued by inhibitor recognition and structure modeling was used. Existing treatments mark anti-inflammatory ways to hinder or slow disease sequence. The recognition of a means to improve axon myelination would present innovative remedial approaches to restrain and probably turn around disease progression. A computational ligand-target docking method was applied to investigate structural composites of the Leucine Rich Repeat and Ig Domain Containing 1 (LINGO1) with three ligands to understand the structural foundation of this protein goal specificity. The following ten residues were conserved for all the three ligands interaction LEU133, ILE134, Pro135, LEU136, ILE155, ILE157, LEU159, ASP160, TYR161, and MET162 which are present in Leucine Rich Repeat 3 and 4 domains. Therefore, these three ligands can be utilized as the potential inhibitors to prevent various neurological disorders and the axonal neuropathies especially the CMT disease. Docking analysis showed that the two important drugs which are widely used have the potential to block the Rho-Rock pathways. Here, we report inhibitors which showed maximum binding affinity for the three most important axonal regeneration inhibitors. However, further studies are required to find the applications of these drugs.

Key words: Demyelination, RTN4, CMT1A, spinal cord injury, ROCK inhibition, neurite growth inhibitors.

INTRODUCTION

A demyelinating disorder is a state of the nervous system in which the myelin casing of neurons is wounded (Perveen et al., 2015). This devastation deteriorates the broadcast of signals in the affected nerves. Sequentially, the decrease in transmission ability causes deficit in sense, movement, thought, or other functions related to nerves. Demyelinating diseases, like multiple sclerosis (MS), are categorized by the damage of the myelin

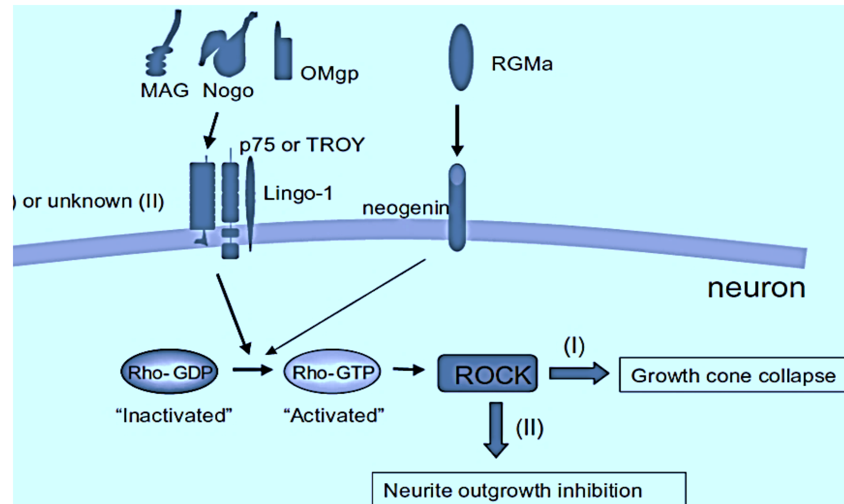

Figure 1. Rho GTP activation leads to activation of ROCK responsible of neurite growth inhibition.

covering around neurons, due to swelling and gliosis in the central nervous system (CNS) and Charcot-Marie-Tooth (CMT) disease (Choi et al., 2015). MS is the supreme common demyelinating disease of the CNS. MS and further demyelinating diseases usually end in vision loss, muscle weakness, muscle stiffness and spasms, loss of coordination, loss of sensation, pain, and changes in bladder and bowel function. Spinal cord injury (SCI) results in damage to axonal tracts that control motor and sensory function. Axons in the spinal cord restore imperfectly, restraining practical retrieval. Wounded neurons in developed organisms are incapable to efficiently regrow their axons after CNS injury. (Filbin, 2003). At least three of these constituents, Nogo-66, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein, either independently or mutually, have been shown to be powerful barrier of neurite outgrowth (Filbin, 2003; He and Koprivica, 2004). All three motion inhibition through the Nogo receptor complex, poised of the ligand-binding Nogo-66 receptor (NgR) and two complementary co-receptors p75 and Lingo-1 that action as a signal-transducing pair on an axon's cell membrane (Barker, 2004; Carim-Todd et al., 2003). Although both NgR and p75 nerve growth factor receptors have fine recognized roles in the context of myelin inhibition; information discovering the role of Lingo-1 are more recent.

The components of myelin with axonal growth inhibitory properties include Nogo-A (Chen et al., 2000; Prinjha et al., 2000), MAG (McKerracher and Rosen, 2015), oligodendrocyte-myelin glycoprotein (OMgp) (Mukhopadhyay et al., 1994), and ephrin-B3 (Benson et al., 2005).

Earlier, Leucine-Rich Repeat and Ig Domain-Containing Nogo receptor-interacting protein (LINGO-1) has been recognized as an *in vitro* and *in vivo* adverse controller of oligodendrocyte distinction and myelination (Mi et al., 2007). LINGO-1 is a negative regulator of neuronal persistence, oligodendrocyte differentiation and axonal outgrowth and renewal, because it relates with assorted growth factor receptors hindering or preventing their action (Figure 1). Steady discoveries achieved *in vitro* and in animal models propose that anti-LINGO-1 therapy may be valuable in neurodegenerative disorders such as MS, Parkinson's disease or essential tremor (ET), and CMT. Furthermore, hereditary and pathological indication deliver a healthy link between LINGO-1 and ET (Agúndez et al., 2015). Myelin-associated inhibitory factors (MAIFs) are inhibitors of CNS axonal renewal following damage. The Nogo receptor complex composed of the Nogo-66 receptor 1 (NgR1), neurotrophin p75 receptor (p75), and LINGO-1, represses axon regeneration upon binding to these myelin components (Shao et al., 2005).

Although neurons in CNS have the capability to restore their axons after damage, but they fail partially since renewal is restricted by growth inhibitory proteins present in CNS myelin (McKerracher and Rosen, 2015). Damage to the CNS in MS seems to be primarily due to continual swelling of the CNS with superimposed bouts of inflammatory activity by the adaptive immune system. The immune arbitrated injured can be augmented by neurodegenerative means in damaged axons as well as

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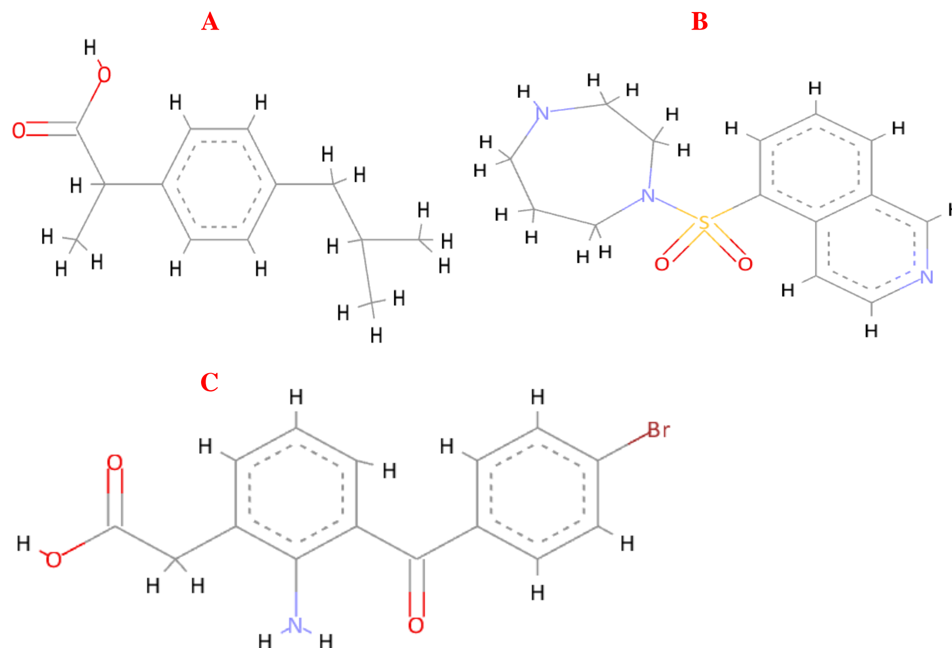

Figure 2. Two-dimensional structure of the ligands: A: $C_{13}H_{18}O_2$; B: $C_{14}H_{17}N_3O_2S$; C: $C_{15}H_{12}BrNO_3$.

anterograde or retrograde axonal or transynaptic deterioration, synaptic pruning and neuronal or oligodendrocyte death. As such, it is highly unlikely that CNS damage can be prevented using only immuno-modulatory drugs (Villoslada, 2016).

In MS, CNS damage is shaped by a complex inflammatory procedure. Even though in the past it was supposed that in the relapsing-remitting phase CNS damage was due only to the presence of inflammatory infiltrates within the MS plaques; in the last decade it has been clearly shown that MS is a disperse disease with swelling, demyelination and axonal loss both in the grey and white matter (Mahad et al., 2015; Ransohoff et al., 2015).

Current treatments therefore target anti-inflammatory mechanisms to impede or slow disease progression. The identification of a means to enhance axon myelination would present new therapeutic approaches to inhibit and possibly reverse disease progression.

METHODOLOGY

Accession of target protein and structure prediction

In the present project, structure forecast, sequence investigation, docking analysis, and relative proteomics investigation were executed on Samsung Electronics Workstation Core-i-5 (Samsung Company, Seoul, Korea). The amino acid sequence of LINGO1 was retrieved for homology modeling. The amino acid sequence was rescued in FASTA arrangement from the Uniprot with the accession number Q96FE5 for previously described gene. The mechanical protein modeling online program swiss modeller was utilized to calculate the 3D structure of LINGO-1 fulfilling spatial restraints. Swiss Modeller and Phyre2 was use to model the structure.

Evaluation of predicted model

Four evaluation tools were used: Rampage (Lovell et al., 2003), 47 ProCheck (Laskowski et al., 1993), 48 Anolea (Colovos and Yeates, 1993), and ERRAT were utilized to evaluate the forecast models. Lastly, the deprived Ramachandran outliers and rotamers were detached by employing WinCoot tool.

Ligand selection

The chemical structures of Ibuprofen, Fasudil and Formanac were obtained from PubChem compound database (Figure 2). It was prepared by ChemBioDraw and MOL SDF format of this ligand was converted to PDBQT file using PyRx tool to generate atomic coordinates.

Target and ligand optimization

For docking investigation, PDB coordinates of the objective protein and ligand molecules were improved by Drug Discovery Studio version 3.0 software and UCSF Chimera tool, respectively. These coordinates had minimum energy and stable conformation (Wass et al., 2010). The chemical structures were estimated to be an efficient drug-like complex subjected to Lipinski's rule of five55 and for their oral bioavailability, supply, metabolism, secretion, and toxicity characteristics of compound and was investigated by applying dmetSAR online server. Eleven mathematical models (Ames toxicity, blood-brain barrier penetration, fish toxicity, aqueous

Table 1. Physical and chemical properties of selected ligands.

Ligand properties	Ibuprofen CID: 3672	Bromfenac CID: 60726	Fasudil CID:3547
Molecular weight (g/mol)	206.2808	334.165	291.369 g/mol
Hydrogen bond acceptor	2	4	5
Hydrogen bond giver	1	2	1
Rotatable bonds	4	4	2
Water solubility	0.0684 mg/ml	0.0126 mg/mL	0.531 mg/mL
Human intestinal absorption	+ (0.9927)	+ (0.9133)	+ (1.0)
logP	3.97	3.4	0.16
Bioavailability	1	1	1
Physiological charge	-1	-1	1
Polar surface area	37.3 Å^2	80.39 Å^2	62.3 Å2
Rotatable bond count	4	4	1
CYP450 2D6 (probability)	Non Inhibitor (0.9231)	Non-inhibitor (0.9003)	Inhibitor
Ames test	Non-amestoxic (0.9894)	Non-ames toxic (0.88)	Non-ames toxic (0.682)
Blood brain barrier (probability)	+ (0.9619)	+ (0.8403)	+ (0.9614)
Rat acute toxicity (LD50, mol/kg)	2.3092	2.5564	2.5211
Biodegradation	Ready biodegradable (0.514)	Not ready biodegradable (0.9618)	Not ready biodegradable (0.9793)

solubility [LogS], honey bee toxicity, carcinogens, acute oral toxicity, Caco-2 permeability, cytochrome P450 2D6 inhibition, rat acute toxicity, and human intestinal absorption) were employed to predict the ADMET possessions of nominated compound. Several poisonousness are expected often and are used in drug designing (Table 1).

Molecular docking analysis

Molecular docking procedures are extensively used for forecasting the binding attractions for a number of ligands. The present effort was to inspect the likelihood of an existing relationship between the investigational bioactivities of the inhibitors under study and the docking scores. In order to get precise results, all the docking trials were completed with the default parameters. PatchDock device was used for docking studies, and crown composites of gene having lowly binding energies were chosen for additional analysis. Dissimilarity was experienced in all the complexes examined with lowest binding energies.

RESULTS AND DISCUSSION

The achievement of the human genome mission has caused a growing amount of new therapeutic targets for drug discovery. At the same time, high-throughput protein distillation, crystallography and nuclear magnetic resonance spectroscopy methods have been recognized and donated to many structural details of proteins and protein-ligand complexes. These advances allow the computational strategies to permeate all feature of drug discovery today (Gohlke and Klebe, 2002; Jorgensen, 2004; Kitchen et al., 2004; Langer and Hoffmann, 2001), such as the virtual screening (VS) techniques (Bajorath, 2002) for hit identification and methods for lead optimization.

Modeling

The purpose of this study was based on the relativeness of LINGO-1 protein with ligands and its bioinformatics analysis to investigate the interactions for curing the axonal neuropathies. The three dimensional structure of the LINGO1 was predicted by using two online tools Swiss modeller and phyre2 (Figure 3).

Evaluation

Estimate tools revealed the consistency and effectiveness of LINGO1 three-dimensional predicted structures (Figure 4). Regions of favored and outlier predicted models were spotted in Ramachandran plot. Only one residue was found in outlier regions. Quality of the expected

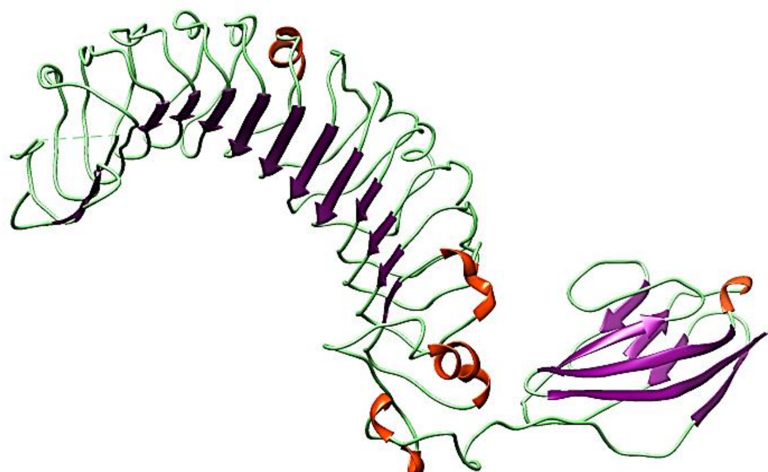

Figure 3. Three-dimensional structure of the Leucine Rich Repeat and Ig Domain Containing 1.

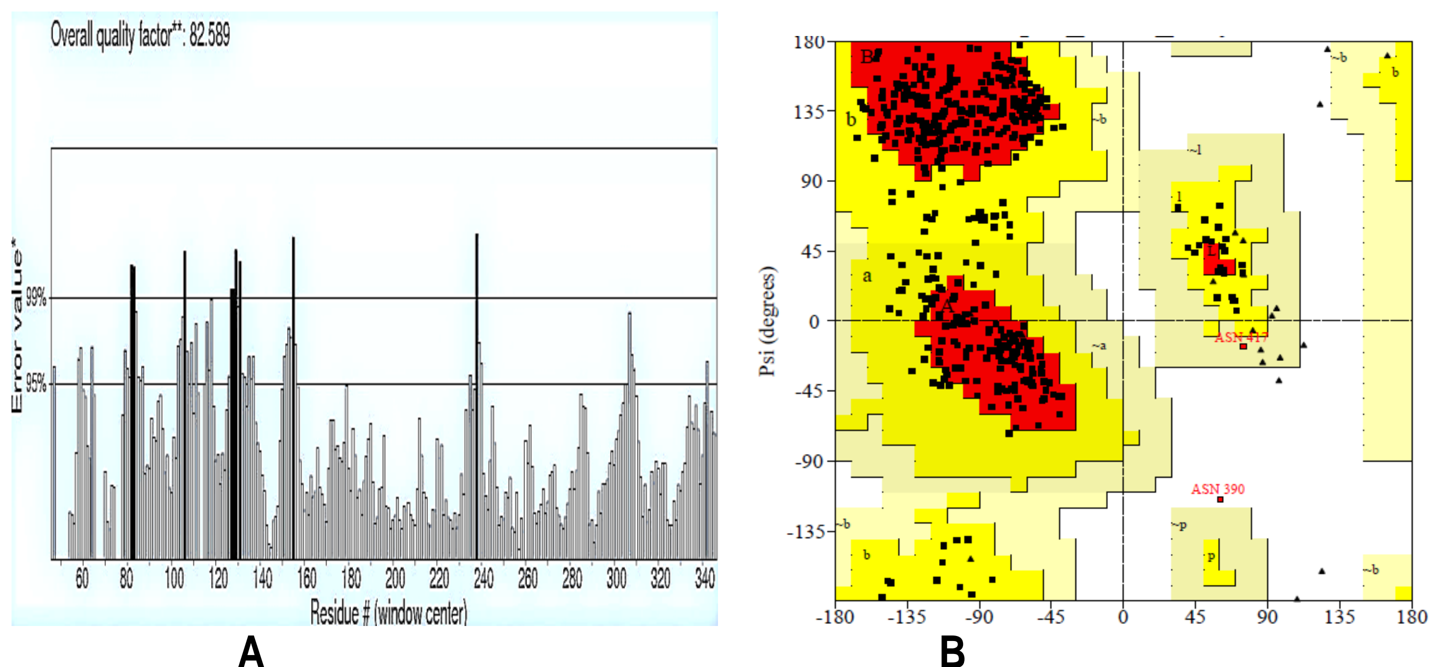

Figure 4. Evaluation of the three-dimensional structure of LINGO1 by (A) ERRAT and (B) Ramachandran plot.

structure was evaluated by Ramachandran plot and ERRAT. The overall quality factor was 83% and only one residue was in the outlier region.

Docking

Drug designing process is not only time taking but also expensive. Therefore, we applied bioinformatics approaches for drug finding procedure. This up-and-coming inclination has huge importance in declining the time essential and amplifying the elected compound with better biological activity and minimal side effects for precise illness targets. The chemical structures are assessed to be an effective drug-like compound subjected to Lipinski's rule of five55 and for their oral bioavailability. Various toxicities are predicted often and are used in drug designing.

Docking of best poses (ligands binding to active sites) of Tirofiban and Amotosalen hydrochloride to CD-61 were

Table 2. Tabular representation of conserved residues in docking analysis.

Protein	Ligand	Conserved residues in 3 ligands	Conserved in two ligands
LINGO1	IBP	LEU133, ILE134, Pro135, LEU136, ILE155, ILE157, LEU159, ASP160, TYR161, MET162	GLY137, LEU158
LINGO1	Bromfenac		GLY137
LINGO1	Fasudil		LEU158

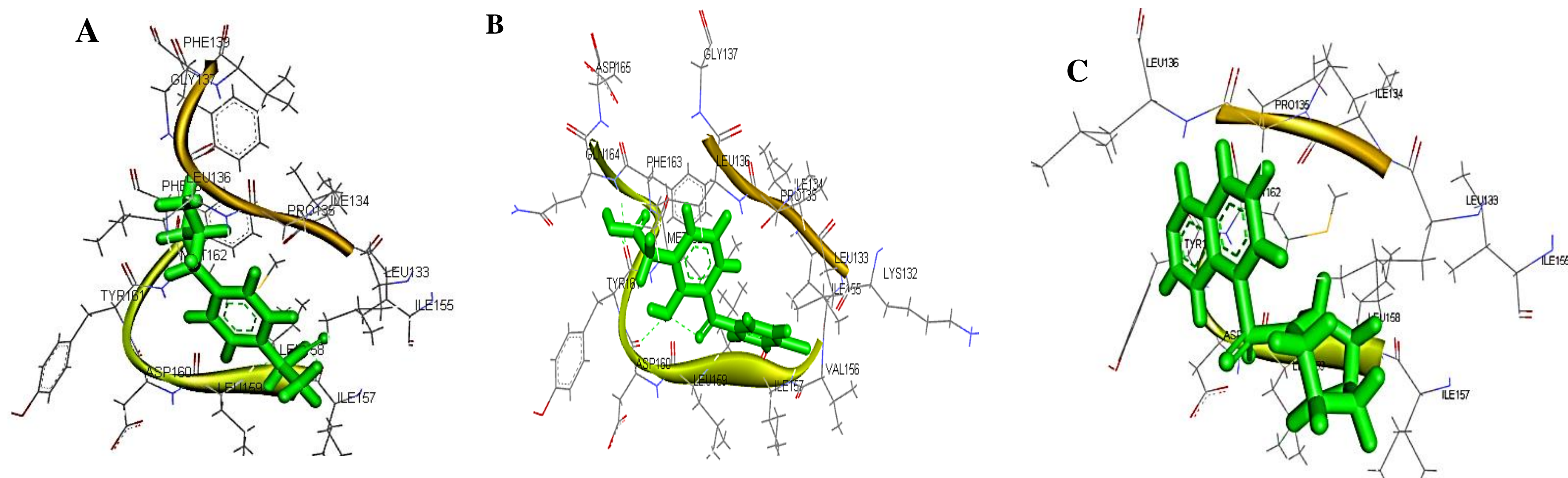

Figure 5. Interaction of Leucine rich repeat and Ig domain containing 1 with three ligands, (A) Ibuprofen, (B) Bromfenac, and (C) Fasudil.

done through Patchdock online docking softwares. PatchDock tool was utilized for docking analysis and top complexes of gene having lowest binding energies were selected for further analysis. From docking analysis, it was observed that the binding residues remains constant in all three ligands which strongly suggest that LINGO1 can be strong candidate to inhibit the RHO/ROCk pathway to enhance the regeneration ability of the axons (Figure 1). Ibuprofen showed the strong binding with LINGO1 at the residues LEU133, ILE134, Pro135, LEU136, GLY137, ILE157, LEU158, and MET162, while Bromfenac showed the binding interaction with LYS132, LEU133, ILE134, PRO135, LEU136, GLY137, ILE155, ILE157, LEU158, LEU159, TYR161, MET162, PHE163, GLN164, and ASP165. The third ligand shows the interaction with the residues at position of LEU133, ILE134, Pro135, LEU136, ILE155, ILE157, LEU158, ASP160, and TYR161.

Deviation was experienced in all the complexes investigated with the lowest binding energies. Possibly, the constancy of the ligand may be because of the allowing of binding likeness. Though, the residues remain steady for production of the complexes with the drugs more preserved. It was observed that Leucine Rich Repeat 3 and 4 was the main domain to show interaction with the ligands (Table 2 and Figure 5).

These are the conserved residues found in the

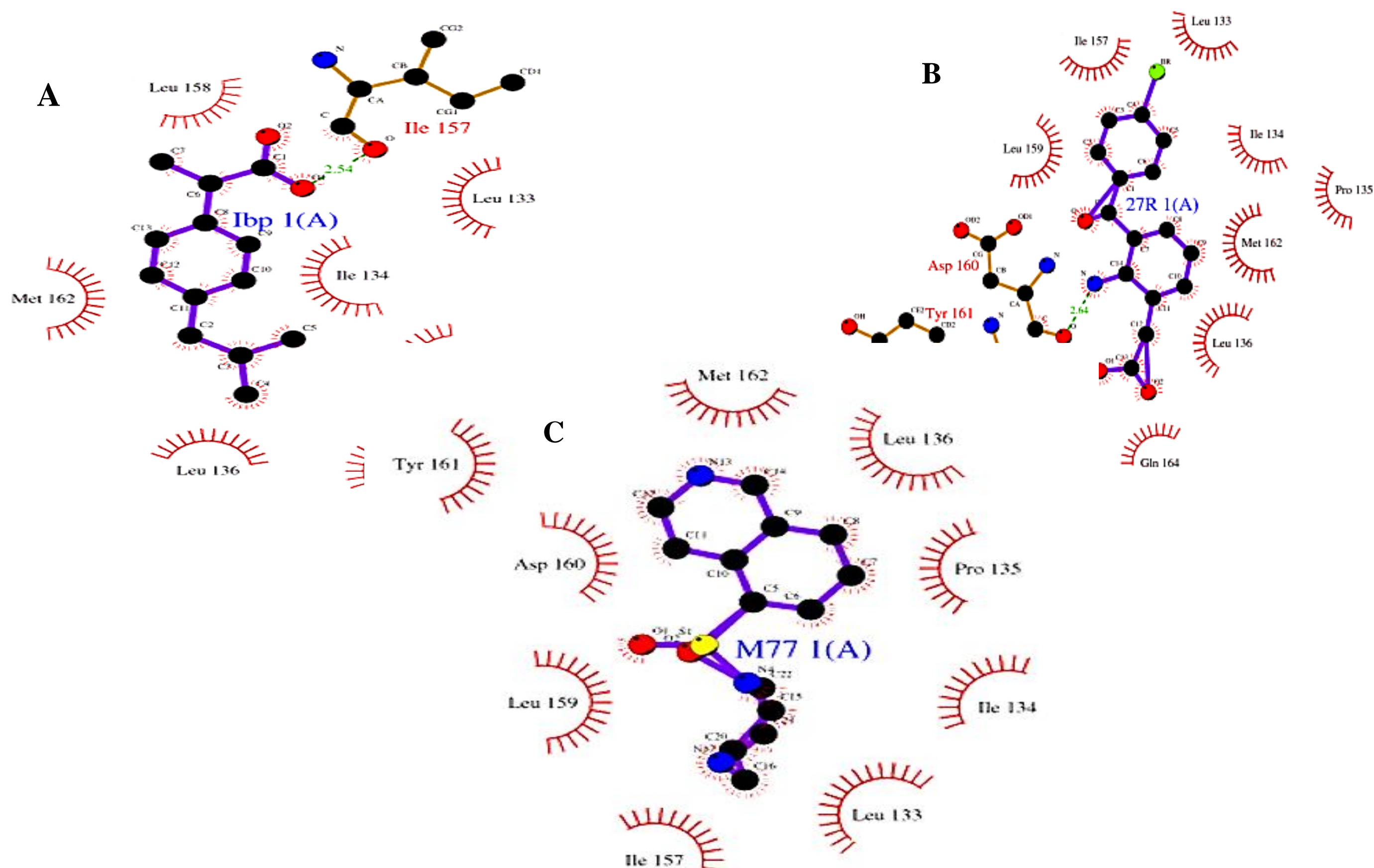

Figure 6. Lig plot interaction of Leucine rich repeat and Ig domain containing 1 with three ligands, (**A**) Ibuprofen, (**B**) Bromfenac, and (**C**) Fasudil.

favored region. In an effort to understand the better relations experiential between ligand and amino acid remainder in the active site of protein, a plot of amino acids-ligand contacts were created employing Ligplot as shown in Figure 6.

Conclusion

Earlier, it was considered that CNS renewal of injured cells is impossible. Existing progress in our thoughtful aspects which limit CNS re-establishment and those which smooth PNS renewal have guide to treatments which let some degree of recover from brain and SCI in animal models. These results open the option of promoting renewal of the injured human CNS.

In wrapping up, the chosen ligands molecules are successful in handling of MS, essential tremer, CMT disease and other neurological disorders for targeting LINGO1. The *in silico* investigation of LINGO1 has higher likelihood and usefulness on the origin of binding energy and other applied parameters. Fasudil is an effective Rho-kinase inhibitor and vasodilator (Barker, 2004; Shibuya and Suzuki, 1993). From the time since it was exposed, it has been used for the treatment of cerebral vasospasm, which is frequently due to subarachnoid hemorrhage (Doggrell, 2005) as well as to pull through the cognitive deterioration seen in stroke victims. It has been found to be efficient for the treatment of pulmonary hypertension (Huentelman et al., 2009). It was verified in February 2009 that Fasudil have the ability to improve recall in normal mice, identifying the drug as a possible treatment for age related or neurodegenerative memory loss. Additional study and production of novel compounds considering these results can anticipate alike reaction and can heal the range of neurological disorders.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

LINGO1, Leucine rich repeat and Ig domain containing 1;

MS, multiple sclerosis; **MAG,** myelin-associated glycoprotein; **CMT,** Charcot-Marie-Tooth disease; **NgR,** Nogo-66 receptor; **SCI,** spinal cord injury; **MAIFs,** myelin-associated inhibitory factors.

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African Journal of Biotechnology

Full Length Research Paper

A new immunoregulatory agent from *Rosa damascena* reduces CD3 expression in human peripheral blood mononuclear cells (PBMCs)

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T-Lymphocytes (T-cells) play a dominant role in the initiation and maintenance of autoimmune inflammatory processes. They become activated by the presentation of antigen such as auto antigen. A main feature of T-cell activation is the production of cytokines. Though, uncontrolled T-cells activation through T-cell receptor (TCR) has been linked to numerous autoinflammatory and autoimmune pathologies. This study investigates the impact of methanol extracts from *Rosa damascena* on suppress T-cells activation. Peripheral blood mononuclear cells (PBMC) were treated by 10, 50,100 µg/ml of *R. damascene* methanolic extracts after activation of T cells with anti- CD3 and anti-CD28. The inhibitory role of methanol extracts of *R. damascena* to suppress the activated T-cells was measured using flow cytometric at three different time points (24, 48, and 72 h). The result showed that the methanol extraction from *R. damascena* petal parts can inhibit the activated T-cells in a dose dependent fashion as we observe reduced levels of cluster of differentiation of CD 3. It is concluded that, extracts of *R. damascena* could be used as a new agent (immunoregulatory) to suppress T-cell activity and treating variety of immune disorders with a low risk of side effects in future.

Key words: T-lymphocytes (T-cells), autoimmune inflammatory, *Rosa damascena*, (CD) 3.

INTRODUCTION

The proinflammatory activity of T-cells could be beneficial to the host during infection. Nevertheless, inappropriate T-cells activation has been associated with some autoimmune and autoinflammatory pathologies. Actually, clinical data shows that T-cells are linked to many autoimmune diseases including arthritis, multiple sclerosis, psoriasis, and lupus (Harrington et al., 2005). It is evident from years of research and development of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, diclofenac sodium and ibuprofen which have had a measure of success in improving pain associated with autoinflammatory disorders and even reducing cellular/hormonal mechanisms involved in this process, through inhibition of initial steps in the biosynthesis

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pathway of prostaglandins (Albert et al., 2002).

With increased use of non-steroidal anti-inflammatory drugs, more side effects have been reported including ulceration, gastric injury, renal damage and cardiac abnormalities which limit their use (Dogne et al., 2006). Similarly Corticosteroids have effective anti-inflammatory activity but have no intrinsic analgesic properties (Coutinho and Chapman, 2011). They are only suitable for treating pain associated with inflammation (Tapiero et al., 2002). Therefore, there is strong interest in the development of new anti-inflammatory drugs, with possibly less side effects, based on natural products

Rosa damascena, also known as Damask rose (Kaul et al., 2000) has been used in traditional medicine for the treatment of several diseases (Boskabady et al., 2011). Herbal active components have various mechanisms of action; finding these active components and their mechanisms in the immune system is remarkably important (Megna et al., 2012). Some of herbal drug functions are characterized by their influence on lymphocytes (Wilasrusmee et al., 2002). *R. damascene* is a perennial erect shrub with aromatic light pink flowers, belongs to genus Rosa species (Rosaceae family) (Kaul et al., 2000).

R. damascena species is native to Damascus, Syria; grown in many countries all over the world for visual beauty and its use in production of fragrances (Boskabady et al., 2011). This plant is a rich source of vitamin C, flavonoids, tannins, carboxylic acids and myrcene (Schiber et al., 2005). In addition to its perfuming properties, it has been traditionally used in medicine to treat chest pains, relieve digestive problems, reduce fever and menstrual bleeding (Yassa et al., 2015). Valuable therapeutic applications of *R. damascena* in modern medicine have been reported as antimicrobial (Basim and Basim, 2003), antitumor (Zu et al., 2010), anti-depressant (Boskabady et al., 2006), anti-oxidant (Baydar and Baydar, 2013), and anti-inflammatory properties (Boskabady et al., 2011; Winther et al., 2005).

Though therapeutic and biological effects of *R. damascene* have been described, its effect on the immune system is not precisely investigated. These studies in traditional and modern medicine suggest that *R. damascene* has diverse effects on the immune system. Investigations over the last 20 years have shown that anti-CD3 drugs effectively treat autoimmune disease in animal models and have also shown promise in clinical trials.

The present study was undertaken to investigate whether alcoholic rose petals extracts has immunoregulatory suppressor effect on T-cell receptor (TCR) activities, as these cells are the major effector cells in cellular and humoral immune responses. Peripheral blood mononuclear cells (PBMCs) were the cells of choice in this study, as these cells are the key immune cell populations that can be easily used to measure the effects of drug treatment especially for researchers, who prefer to use human samples.

MATERIALS AND METHODS

Plant material and preparation of extract

Flowers of *R. damascena* were collected early morning in April 2016, during the harvest season from Al Hada local farm (Taif-Saudi Arabia). The flowers were taxonomically identified and authenticated by Dr. Hadeer Darwesh, Biotechnology Department, Taif University. The total ethanol extracts was prepared by Soxhlet extraction method.

Fresh petal parts of the flowers were extracted with 100% methanol at ratio 8:1. The process of extraction carries on for 6 h at 60°C. The crude methanol extract was obtained after evaporating the ethanol to dryness at 40°C under reduced pressure. The crude extract was collected and stored at 20°C for further studies, and the percentage yield of the extract was calculated. 100% Dimethyl sulfoxide (DMSO) was used later to dissolve the dried extracts and prepare in different concentrations.

Antibody coating of the plate

24 well plates (Coster, Corning, NY) were pre-coated with 1 µg/ml of mouse anti-human CD3 mAb (BD Bioscience) in sterile (phosphate-buffered saline) PBS was prepared, a 50 µL of the antibody solution was dispensed to each well of the 24 well plate. The plates were incubated at 37°C for 2 h, and the solution was removed and rinsed twice with 200 µL of sterile PBS to remove all unbound antibody from each well before adding cells (Protocol was adapted from eBiosciences).

Human PBMCS separation and culture

Blood samples were obtained from healthy donors. The mean age of the patients was 49 years. 15 mL blood was drawn in a heparin tube (Becton Dickinson, Lincoln Park, NJ, USA). PBMC were isolated by mixed 1:1 with PBS and 20 mL histopaque was carefully carried out under layered in 50 mL tubes, followed by centrifugation at 1800 x g at room temperature for 20 min. The PBMCs were removed using 5 ml pipette, washed twice in Hank's Buffered Saline and cells were centrifuged at 400 g for 10 min.

The cell pellet was finally washed twice with RPMI 1640 (Gibco BRL, Uxbridge, UK) and re-suspended in medium consisting RPMI 1640, supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 25 mM HEPES buffer, 50 u/ml penicillin and 50 µg/ml streptomycin. (All from Sigma Chemical Co., Poole, UK).

T-lymphocyte activation and rose extracts treatments (effects of rose extracts on t-cell activation)

One (1) mm aliquots of the PBMC suspension at a concentration of $1x10^6$ cells/mL were added to 24 well plates, pre-coated with anti-human CD3 mAb and for each condition, triplicate wells were used. Soluble anti-CD28 was added to cells at 1 µg/mL. Cells were treated with 10 µl of rose at various concentrations (0.5, 0.005, 0.00005 µg/ml) and plates were placed to humidified at 37°C, 5% CO_2 incubator. Cells were cultured at three different time points (24, 48, and 72 h). Control cells were incubated with 10 µl of RPMI 1640 for untreated cells, and with 10 µl of 100% DMSO.

Flow cytometric analysis

Immunofluorescence staining was performed according to the

method described by Beutner (1961). In brief, cells were harvested, washed in PBS containing 0.5% (v/v) BSA, and re-suspended in PBA buffer (PBA was prepared by mixing PBS and 0.5% BSA to 0.1% Sodium azide). Cells were then mixed with mouse anti-human CD25 PE–, CD4 FITC–, CD69 PCS–, and CD3 ECD–conjugated antibodies (BD Pharmingen) (R and D Systems, Abingdon, UK) and incubated on ice for 30 min in the dark.

Cells were then washed twice with PBS containing 0.5% BSA, re-suspended in 0.5% (w/v) paraformaldehyde fixative, and analysed by flow cytometry (FC500 flow cytometer, Beckman Coulter, Brea, CA).

Data analysis

Statistical analysis was achieved using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) was use to analyze data. The p-values less than 0.05 were considered to indicate significant differences. Results are expressed as a mean ± standard deviation (SD).

RESULTS

This study was undertaken to evaluate methanol *R. damascena* petal parts extracts effects on suppressing the activated T-lymphocytes (T-cells), by investigating the expression level of cluster of differentiation (CD) 3 that was expressed on the surface of T-cells. To investigate the likely inhibitory role of methanol extracts of *R. damascena* and their ability to suppress the activated T-cells flow, cytometric analysis was performed to investigate the expression level of CD3 T-cell co-receptor. The harvested cells were obtained after activation of T-cells and treat them with different concentration 100, 50 and 10 µg/ml methanol extracts of *R. damascena*, analyse at three different time points (24, 48, and 72 h). The level of CD3 expression by the treated cells were compared with those of unactivated (untreated) and activated DMSO treated cells.

According to the results, the expression level of CD3 increased in control cells treated with 100% DMSO alone after 2, 48, and 72 h (Figures 1, 2 and 3, respectively) as compared to unactivated (untreated) cells, which showed significantly decreased in the expression of CD3 ($p < 0.0001$). However, the expression of CD3 decreased when the cells were treated with methanol extraction from *R. damascena* petal parts, demonstrating that the extracts could suppress the activated T cells in a dose-dependent manner significantly.

When the methanol extraction from *R. damascena* petal parts concentration rise to 100 µg/ml, the expression of CD3 being equally as unactivated (untreated) cells was significantly decreased ($p < 0.0001$) at all point in time when compared to cells treated with 100% DMSO alone. Treatment with 50 µg/ml concentration result in significant difference in CD3 expression as compared with that of 100% activated DMSO treated cells (24 h $p<0.05$, 48 h $P<0.0001$ and 72 h $P<0.01$). Treatment with 10 µg/ml concentration from *R. damascena* extracts at 24 and 72 h showed no significant difference in the expression of CD3 compared with 100% activated DMSO treated cells, but the expression of CD3 was significantly decreased at 48 h ($P<0.01$) indicating that at this point in time, this concentration provide significant suppress to activated T-cells as compared to other time points.

In other words, the result suggested that the methanol extraction from *R. damascena* petal parts can inhibit the activated T-cells as shown in Figures 1, 2, and 3 compared with 100% activated DMSO treated cells alone, the activity of the methanol extraction from *R. damascena* petal parts in low dose is less effective. But with extracts concentration increasing to 100 µg/ml, it could significantly suppress the activated T-cells.

DISCUSSION

R. damascena is an ornamental plant, a member of Rosaceae family with more than 200 species and 18,000 cultivars around the world. Besides its perfuming effect, many pharmacological properties have been reported by several other researcher (Boskabady et al., 2011; Sedighi et al., 2014), which is of great interest in *R. damascena* because of its stated valuable effect in a variety of immune conditions and also for suppression of the immune system particularly lymphocytes.

R. damascena contains several compounds such as flavonoids, kaempferol, geraniol, citranellol, eugenol, linalool, nerol, myercene and vitamin C that have applications in several therapeutic areas including HIV (Mahmood et al., 1996), diabetes (Gholamhoseinian et al., 2009), depression (Boskabady et al., 2006), cancer (Venkatesan et al., 2014), inflammation (Hajhashemi et al., 2010), and several infectious areas (Basim and Basim, 2003).

Exploration of the methanol *R. damascena* petal parts extracts effect on the expression of CD3, presented on the surface of activated lymphocyte (T cells) showed decreased in CD3 expression in a dose-dependent manner. Higher concentrations (100 and 50 µg/ml) of *R. damascena* petal parts extracts have a suppressive effect on activated T cells at all point in time (24, 48, and 72 h) as shown in Figures 1, 2, and 3 compared with 100% DMSO alone, the suppressive activity of the methanol extraction from *R. damascena* petal parts in low dose (10 µg/ml) is less effective.

Briefly, our results have revealed that *R. damascena* ingredients in alcohol extraction suppress the activated T-cell. The suppressive properties of *R. damascena* in cell growth have been studied several times and it has been found that *R. damascena* could be used in inhibition of cancer cell development which induces the apoptosis and increases the expression of apoptotic protein (Elson, 1995).

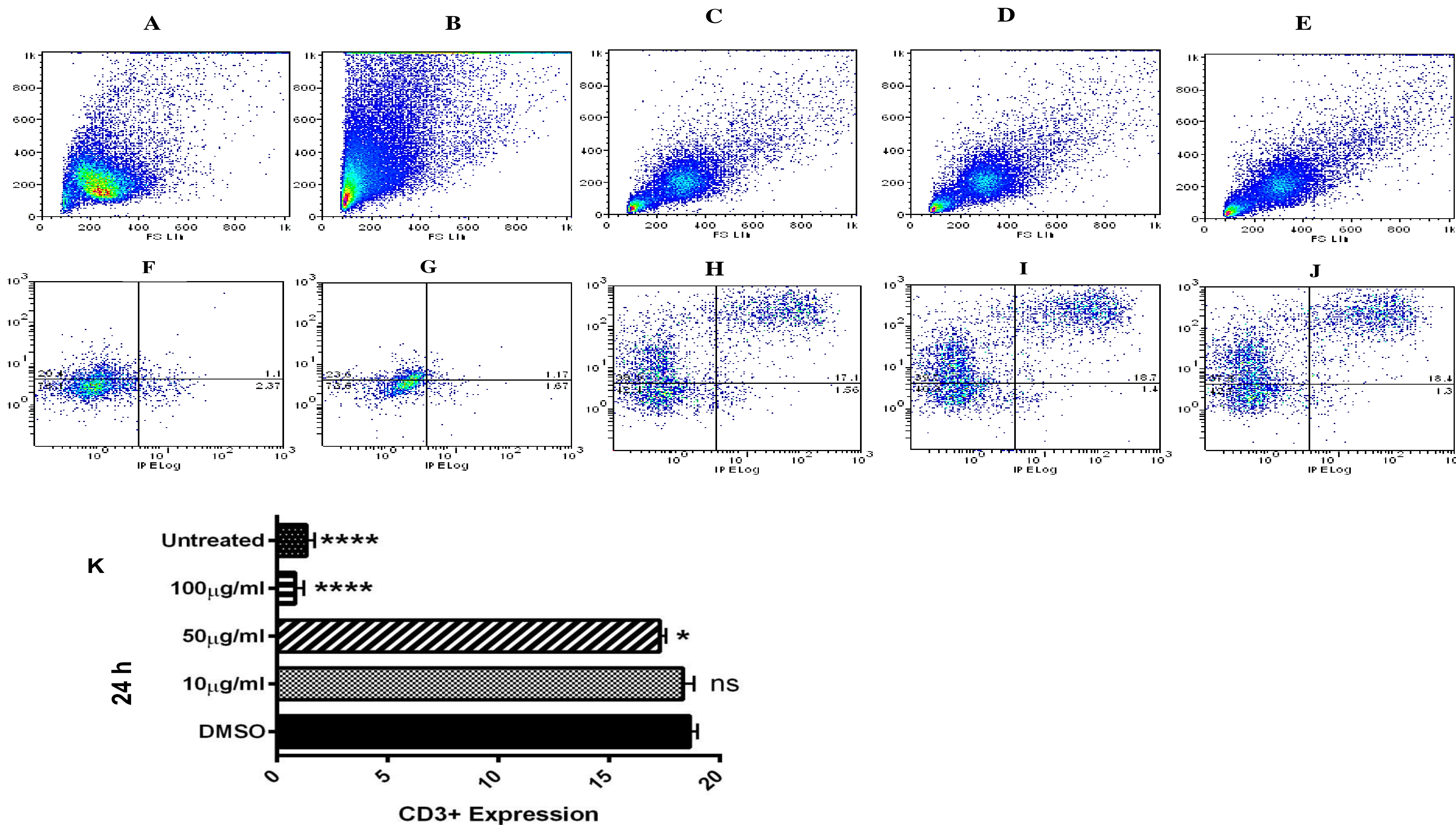

Figure 1. Assessments of the CD3 expression in PBMCS treated with methanol extracts of *R. damascena* petal parts by cytometric analysis after 24 h. (A, B, C, D and E) show the forward scatter (FSC) versus side scatter (SSC) dot blot. (F, G, H, I and J) y-axis shows PCS fluorescence log and the x-axis shows IPE log fluorescence. The cells were treated as follows: A &F untreated cells; B&G cells treated with 100 µg/ml methanol extracts of *R. damascena*, C&H cells treated with 50 µg/ml methanol extracts of *R. damascena*, D&I cells treated with 10 µg/ml methanol extracts of *R. damascena*, E&J) Negative control cells treated with DMSO. All treated cells were incubated for 24 h and then harvested and washed in PBS, and stained using conjugated antibodies [mouse anti-human CD3 PE–, and CD28 PCS (BD Pharmingen) (R&D Systems, Abingdon, UK)] for 30 min on ice in the dark. Cells were washed twice, and analysed by flow cytometry (FC500 flow cytometer, Beckman Coulter, Brea, CA). K) Multiple comparison of the suppress effects of different concentration of 100, 50 and 10 µg/ml methanol extracts of *R. damascena* compared to the effect of 100% DMSO treatment; data were analysed by One-way ANOVA and P values are shown where the difference between responses of different treatment relative to the control were determined to be statistically significant: ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. These results are representative of 3 independent repeats. FS Lin, forward scatter; SS Lin, side scatter.

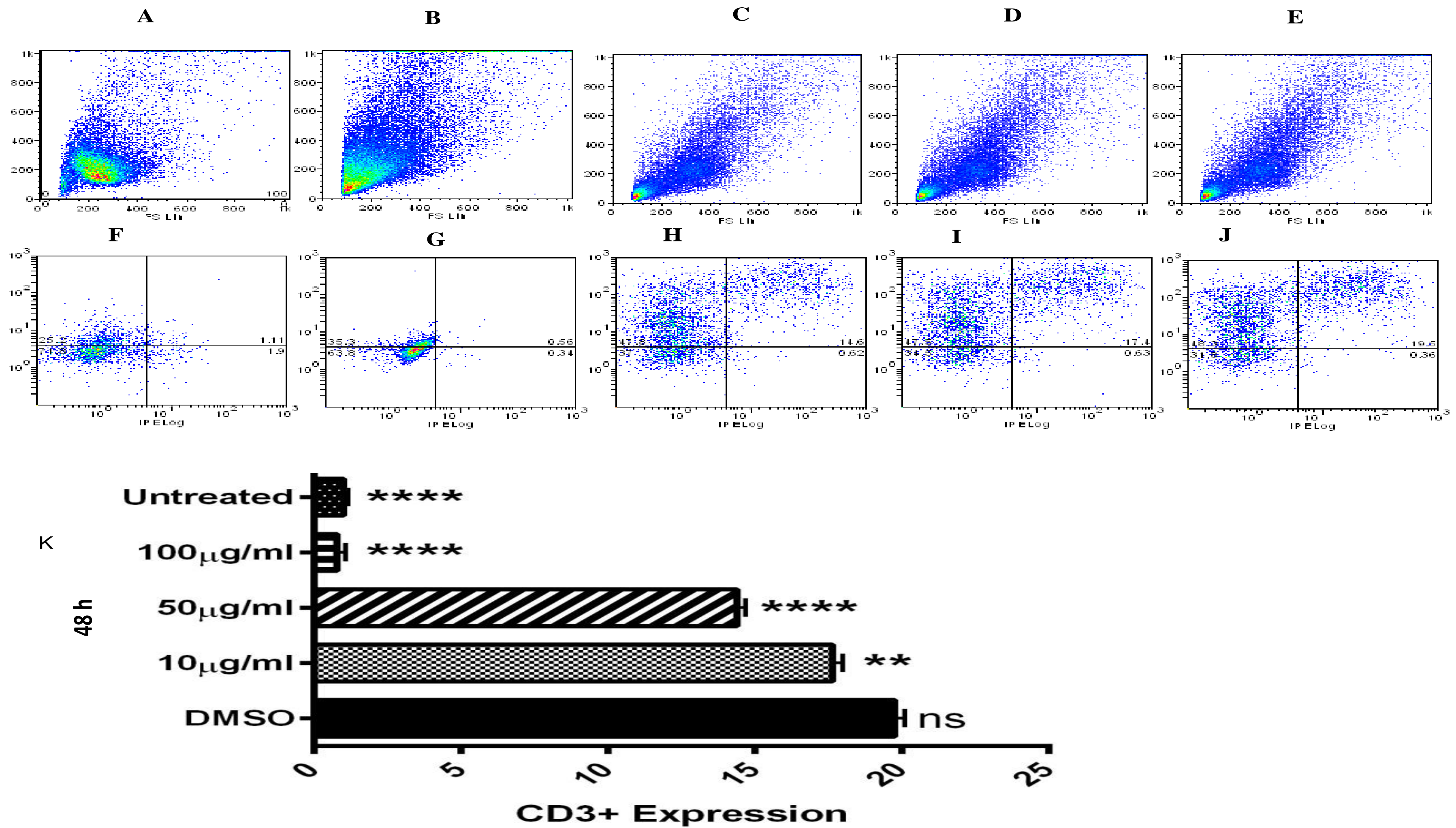

Figure 2. Assessments of the CD3 expression in PBMCS treated with methanol extracts of *R. damascena* petal parts by cytometric analysis after 48 h. (A, B, C, D and E) show the forward scatter (FSC) versus side scatter (SSC) dot blot. (F, G, H, I and J) y-axis shows PCS fluorescence log and the x-axis shows IPE log fluorescence. The cells were treated as follows: A &F, untreated cells; B&G, cells treated with 100µg/ml methanol extracts of *R. damascene*; C&H, cells treated with 50 µg/ml methanol extracts of *R. damascene*; D&I, cells treated with 10 µg/ml methanol extracts of *R. damascena*; E&J, negative control cells treated with DMSO. All treated cells were incubated for 48 h and then harvested and washed in PBS, and stained using conjugated antibodies [mouse anti-human CD3 PE–, and CD28 PCS (BD Pharmingen) (R&D Systems, Abingdon, UK)] for 30 min on ice in the dark. Cells were washed twice, and analysed by flow cytometry (FC500 flow cytometer, Beckman Coulter, Brea, CA). K) Multiple comparison of the suppress effects of different concentration 100, 50 and 10 µg/ml methanol extracts of *R .damascena* compared to the effect of 100% DMSO treatment, data were analysed by One-way ANOVA and P values are shown where the difference between responses of different treatment relative to the control were determined to be statistically significant: ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. These results are representative of 3 independent repeats.

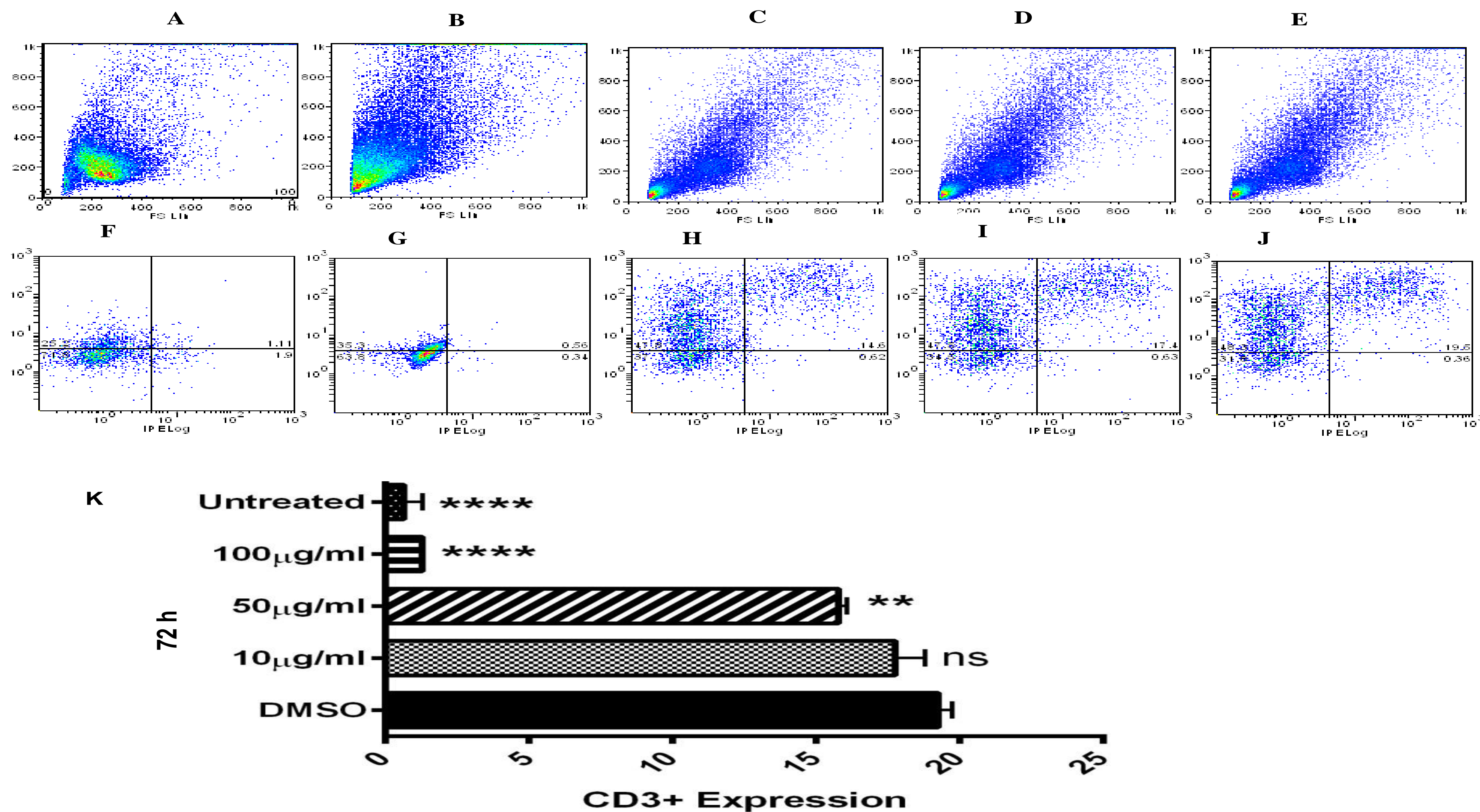

Figure 3. Assessments of the CD3 expression in PBMCS treated with methanol extracts of *R. damascena* petal parts by cytometric analysis after 72 h. (A, B, C, D and E) show the forward scatter (FSC) versus side scatter (SSC) dot blot. (F, G, H, I and J) y-axis shows PCS fluorescence log and the x-axis shows IPE log fluorescence. The cells were treated as follows: A &F, untreated cells; B&G, cells treated with 100 µg/ml methanol extracts of *R. damascena*; C&H, cells treated with 50 µg/ml methanol extracts of *R. damascena*; D&I, cells treated with 10 µg/ml methanol extracts of *R. damascena*, E&J) Negative control cells treated with DMSO. All treated cells were incubated for 72 h and then harvested and washed in PBS, and stained using conjugated antibodies [mouse anti-human CD3 PE–, and CD28 PCS (BD Pharmingen) (R&D Systems, Abingdon, UK)] for 30 min on ice in the dark. Cells were washed twice, and analysed by flow cytometry (FC500 flow cytometer, Beckman Coulter, Brea, CA). K) Multiple comparison of the suppress effects of different concentration 100, 50 and 10 µg/ml methanol extracts of *R. damascena* compared to the effect of 100% DMSO treatment, data were analysed by One-way ANOVA and P values are shown where the difference between responses of different treatment relative to the control were determined to be statistically significant: ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. These results are representative of 3 independent repeats.

Conclusion

It might be concluded that the methanol extracts of *R. damascena* petal parts was obtained through Soxhlet extraction method. The results from the *in vitro* proved that the extracts could suppress the activated T-cells, as we observe reduced levels of CD 3.

It is inferred that extracts of *R. damascena* can be used as a new agent (immunoregulatory) suppressor T-cell activities. Moreover, this could restore cellular immunity, which would have valuable applications as it offers an effective and safe strategy for treating variety of immune disorders, with a low risk of side effects in future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of hygienic quality of ferment of local beer "dolo" used as condiment in Burkina Faso

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***Rabilé* is a ferment of local beer called "*dolo*" in Burkina Faso. It is used for local beer production and as condiment in sauces. Its process of production could be a source of contamination. So the quality of ferment needs to be guaranteed for consumers' health. The aim of the study was to control the hygienic quality of local ferment consumed in Burkina Faso. A total of 70 samples were purchased in seven localities of Burkina Faso. The standard plate count method was used for microbial analysis. The results show that aerobic mesophilic flora varied from 8.34 ± 0.72 to 10.07 ± 0.51 $\log_{10}$ UFC.g^{-1}; yeast and mold from 7.24 to 8.28 $\log_{10}$ UFC.g^{-1}; *Staphylococcus aureus* from 4.08 to 4.76 $\log_{10}$ UFC.g^{-1}; *Enterococci* from 3.47 to 4.61 $\log_{10}$ UFC.g^{-1} and total coliform from 2.12 to 2.32 $\log_{10}$ UFC.g^{-1}. *Salmonella* spp. and *Shiguella* spp. were not detected in any of the samples. So, 100% of the samples from Ouahigouya were contaminated with sulphite reducing bacteria (BRS). All the samples analyzed were contaminated by *S. aureus* and *Enterococc*i. The hygienic control of the ferment showed that the contamination caused by food borne pathogens can lead to food poisoning. Pearson correlation matrix of microorganisms' distribution in the samples showed highest positive correlation between Enterococci and sulphite reducing bacteria at r = 0.888. Analysis of principal components exhibited the variability of microbial groups with 94.312% cumulative values of the variance and Eigen values ranging between 0.492 and 3.695. The data suggested that traditional beer ferments samples collected were not suitable in quality and there is need to ameliorate the process of production.**

Key words: Local ferment, dolo, hygienic quality, food, Burkina Faso.

INTRODUCTION

Traditional beer ferment of Burkina Faso is called "rabilé" in Mooré, "dambilé" in Dagara, "yantoro" in San, "li dabili" in Gourmatché, "seinbié" in Gouroussi, "kuinguié" in Bobo, "yiibou" in Bissa, also called Kpètè-Kpètè or Otchè

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in Benin, Umusemburo in Rwanda (Djêgui et al., 2015; Keita et al., 2016; Lyumugabe et al., 2014; N'tcha et al., 2016). It is used for cooking traditional beer and for preparation of certain sauces in some communities of Burkina Faso.

The local beer is cooked in Burkina Faso using *Sorghum bicolor* L, *Setaria italic* or *Zea mays*. The traditional process of local beer production involves three principal steps: malting, brewing and fermentation process. However, variations can occur according to ethnical group, the producers and the locality (Coulibaly et al., 2014; Songre-Ouattara et al., 2016). In African countries, sales of the local beer constitute an important source of money for the sellers. Moreover, local beer is used in some ceremonies like funeral, wedding and baptism (Maoura et al., 2005; Djegui et al., 2015; Keita et al., 2016).

Studies carried out by the different authors (Glover et al., 2009; Sawadogo-Lingani et al., 2010; Djegui et al., 2015; N'Guessan et al., 2015) have shown that *Lactobacillus* sp., *Leuconostoc* sp. and *Saccharomyces cerevisae* are principal microorganisms found in the fermentation of traditional beer. Sawadogo-Lingani et al. (2008), Kayode et al. (2012) and N'tcha et al. (2016) have revealed probiotic property of lactic acid bacteria isolated from fermented local sorghum beer. The ferment constitutes interesting nutriments source. Wijeyaratne and Jayathilak (2000) demonstrated that *Candida* sp. contains vitamin B (riboflavin (0.231 mg/g), and thiamin (0.178 mg/g). The percentage of its protein ranges from 50 to 60.31% (Dimova et al., 2010; Mondal et al., 2012; Ouédraogo et al., 2012) in the ferment source. Ouedraogo et al. (2017) revealed that *Candida utilis* NOY1 contains 54.8 ± 0.12% of protein. Thus, people in Burkina Faso used local ferment as condiment in sauces and to dress some dishes of chicken or pork meat.

Mainly studies have been done on the processing of local beer and fermentation, like technological property of starters from beer and fermentation of sorghum and beer qualities (N'Guessan et al., 2010; Abdoul-latif et al., 2012; Abdoul-latif et al., 2013; N'tcha et al., 2015; Djegui et al., 2016; Songre-Ouattara et al., 2016). It is observed that there is lack of information on hygienic and microbial qualities of local ferment used as starter for fermentation or condiment in sauces. The process used for local fermentation is sometime affected by hygienic conditions and it could be contaminated with food borne pathogens. The source of contamination could come from practice, tools, and producers themselves.

This study aimed to evaluate the hygienic quality of ferment extracted from local beer used in dishes in Burkina Faso. Quality hygiene can prevent certain contamination from pathogenic microorganism and thus enhance quality and safety.

MATERIALS AND METHODS

The experiment was conducted in the Laboratory of Biotechnology, Biological Food and Nutrition Sciences at the Research Center in Biological Food and Nutrition Sciences (CRSBAN / University Ouaga I Pr Joseph KI-ZERBO). The seven ways of processing of local beer are followed in the sites of production like Ouahigouya, Fada N'Gourma, Réo, Bobo Dioulasso, Grarango and Tougan Gaoua in Burkina Faso.

Process of traditional beer and ferment production

An investigation was realized with 70 traditional beer producers and diagram production of traditional beer and ferment (Table 1).

Sample collection

The ferment of local beer was collected from 70 (seventy) sellers of local beer in 7 (seven) localities Ouahigouya, Fada N'Gourma, Réo, Bobo Dioulasso, Grarango, Tougan Gaoua (Table 1). From each producers, one hundred grams (100 g) of local ferment was sampled and maintained at 4°C in isothermal box for laboratory analysis.

Microbiological analysis

Samples microbiological analysis was carried out to determine the levels of aerobic mesophilic bacteria; yeast and molds, total coliform, thermo tolerant coliform including *Escherichia coli*, *Staphylococcus aureus*, *Enterococci*, sulphite reducing bacteria, *Salmonella* spp. and *Shiguella* spp. by standards microbiology system using the methods of De Souza et al. (2011) and Somda et al. (2014). The prevalence rates and levels of presumptive were determined in 70 samples.

Cell numeration

For each sample, 10 g was mixed with 90 mL trypton-salt water (0.85% w/v) and stored at ambient temperature for 30 min. Afterwards, tenfold serial dilutions were made for each suspension and 0.1 mL diluted suspension was used to inoculate specific culture media for microbial isolation and enumeration as recommended by the French Association of Normalization (AFNOR, 2009). The total number of microorganisms expressed as colony forming unit (cfu) per gram of sample was determined by standard plate count. The microorganisms were enumerated under the following conditions:

1. Total aerobic mesophilic bacteria with plate count agar (PCA) after 24 h incubation at 30°C.
2. Total coliforms and thermotolerant coliforms on Eosin Methylen Blue medium (EMB) after 24 h incubation at 37 and 44°C, respectively.
3. Yeasts and molds were counted in a Sabouraud after 3 to 5 days of incubation at 30°C, under aerobic condition.
4. Sulphite reducing bacteria (SRB) by most probably number (MPN) with tryptone-sulfite neomycin broth after 20 h incubation at 44°C.
5. *Salmonella* sp. and *Shigella* sp. were researched in a SS medium after 3 to 5 days of incubation at 37°C.
6. *S. aureus* by counting chapman coagulase positive colonies after 24 h incubation at 37°C.
7. *Enterococci* by counting Enterococcel agar after 24 h incubation at 37°C.

Standard identification methods including Gram stain morphology, colony morphology, production of catalase or oxidase, and lactose

Table 1. Different process steps of traditional beer and ferment production.

Process according to ethnical group	Process steps	Role of steps	Locality
Mossi	a, b , c, e, f, g, h, i, j, k, l	To obtain alcoholic drink and traditional ferment	Ouahigouya about 110 of km Ouagadougou
Bissa	a, b , c, e, f, g, h, i, j, k, l	To obtain alcoholic drink and traditional ferment	Garango about 200 km of Ouagadougou
Dagara	a, b , c, e, f, g, h, i, j, k, l	To obtain alcoholic drink and traditional ferment	Dissine about 389.2 km of Ouagadougou
Samo	a, b , c, e, f, g, h, i, j, l	To obtain alcoholic drink and traditional ferment	Tougan about 214.3 km of Ouagadougou
Bobo	a, b , c, e, f, g, h, i, j, k, l	To obtain alcoholic drink and traditional ferment	Bobo-Dioulasso about 351 of Ouagadougou
Gourmatche	a, b , c, e, f, g, h, i, j, k, l	To obtain alcoholic drink and traditional ferment	Fada N'Gourma about 221 km of Ouagadougou
Gouroussi	a, b , c, e, f, g, h, i, j, k, l	To obtain alcoholic drink and traditional ferment	Reo about 129.6 km of Ouagadougou

a, Soaking; b, germination; c, drying in the sun; d, mashing and added vegetal mucilage; e, decantation/filtration and residues cooking; g, decantation/filtration; h, mixing with surviving collected; i, cooking; j, cooling; k, alcohol fermentation; l, decantation.

fermentation were used for microorganisms phenotypical identification.

Microbial quality of traditional beer ferment was assayed using the critical limits of French Association of Normalization (AFNOR).

Statistical analysis

Descriptive statistics were established to report the variability of the different parameters involved in the evaluation of the traditional fermentation. Microbial counts were transformed into decimals logarithmic. Log transformed counts of microbiological indicators data were analyzed using factorial analysis of variance between means of microorganism's number with respect to different sources. Correlation between different microbial indicators counted was determined. A p-value of < 0.05 or 0.0001 was considered statistically significant. Associations between microbial groups were performed through Pearson correlation at 5%. The Pearson's correlation coefficients between variables log number of different groups of tested microorganisms in traditional beer ferments samples were calculated. Principal component analysis was performed in order to identify the microbial groups or localities, which are best represented.

RESULTS AND DISCUSSION

Diagram of production of traditional beer and ferment

Traditional beer production is a complex, long process and varies according to the producers, geographic locality and ethnical group. It included mainly three steps such as: malting, brewing and fermentation. The different steps of process used for traditional beer and ferment production are synthetized in Figure 1.

The figure demonstrates the different steps in the local beer production. A similarity was found in the principal points of preparation: malting, brewing and fermentation. The main difference based on ethnical group essentially concerned the frequency of filtration and cooking during mixing and fermentation. Moreover, the mixture of some mucilages of *Abelmochus esculentus*, *Curculigo pilosa*, *Gladiolus klattianus* enzymes or leaves of *Adansonia digitata*, *Boscia senegalensis*, *Grewia bicolor* differed according to the producer. There is no significant difference between the producers of the different localities. The malting process involves soaking, germination and sun drying. Lyumugabe et al. (2012) and Abdoul-latif et al. (2013) showed that malting process allows the activation of synthesis of hydrolytic enzymes (α-amylases, β-amylases, proteases, etc.). During the brewing process, starch and proteins are converted into fermentable sugars and peptides. The alcohol fermentation lasts between 8 and 12 h, and it is produced by the old drink or the old leaven.

This diagram of production has concordance with some authors like Lyumugabe et al. (2012), Abdoul-latif et al. (2013) and Coulibaly et al. (2014) who found three principal steps during beer cooking. Some authors like Abdoul-latif et al. (2013), Atter et al. (2014) and Coulibaly et al. (2014) have added that the disadvantage of "dolo" preparation is lack of time.

Numeration of isolated microorganisms

The main microbial groups were counted and converted to log_{10} $CFU.g^{-1}$ in analysis of local starter (Table 2). All the 70 samples of local ferment did not contain *Salmonella* sp. and *Shigella* sp. However, all samples contained aerobic mesophilic bacteria, yeast and mold, *S. aureus*, *Enterococci* and sulphite reducing bacteria. Aerobic mesophilic bacteria count ranged from 8.34 to 10.07 log_{10} ($CFU.g^{-1}$); yeast and mold from 7.24 to 8.28

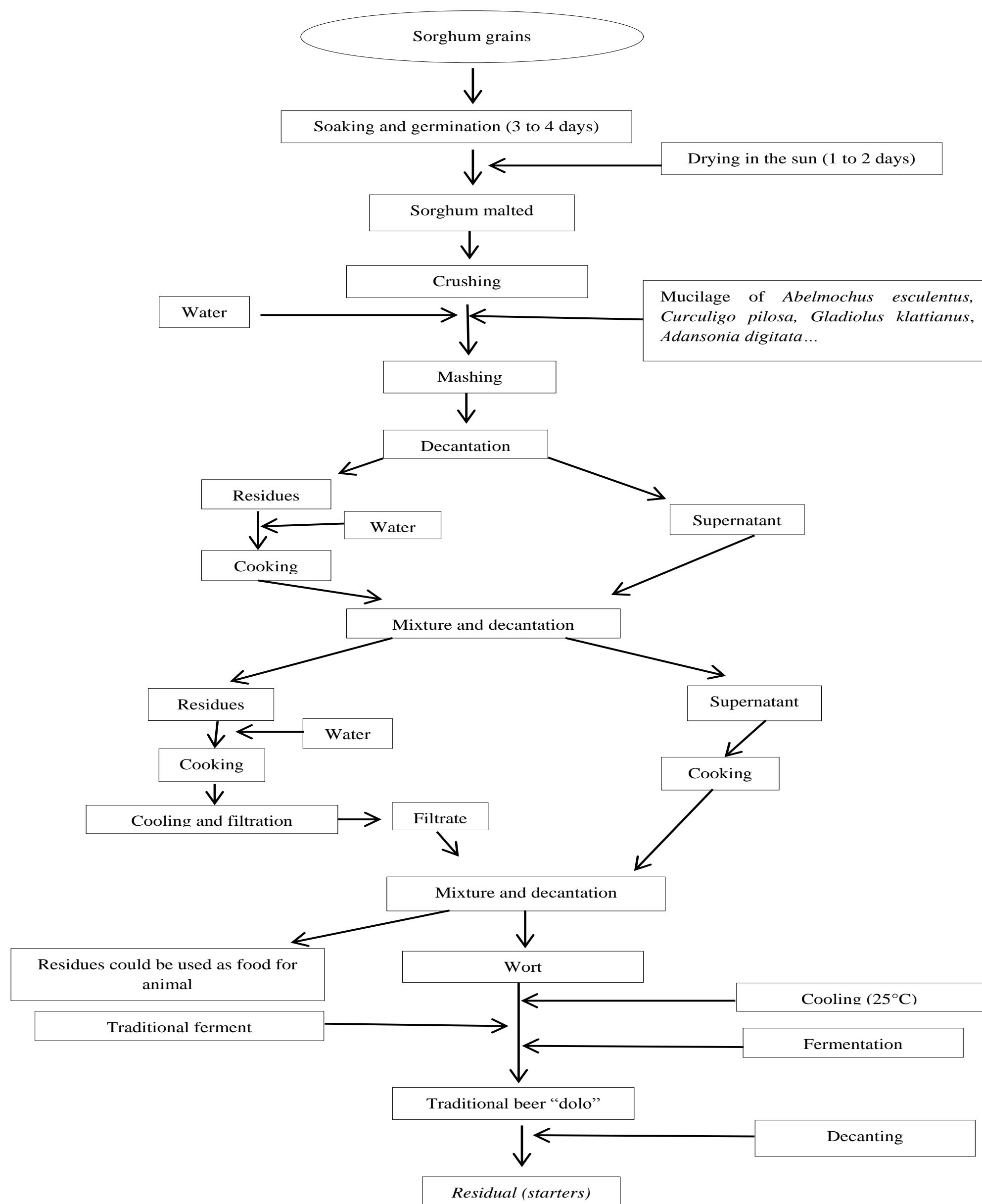

Figure 1. Diagram of production of local beer and starters.

Table 2. Microbial profile in traditional ferment.

Locality	Fada N'Gourma	Ouahigouya	Tougan	Réo	Garango	Gaoua	Bobo Dioulasso	p-value
	Range count of microorganisms per g for all sample, in $\log_{10}$ ($CFU.g^{-1}$)							
Mesophilic aerobic bacteria	9.21	9.38	10.07	8.87	8.42	8.34	9.32	<0.0001
Yeasts and molds	8.23	7.82	8.28	7.76	7.24	7.27	7.9	<0.0001
Staphylococcus aureus	4.11	4.52	4.76	4.4	4.41	4.08	4.71	<0.0001
Total Coliform	2.14	2.32	nd	nd	2.22	nd	nd	
Thermo tolerant coliform	nd	nd	nd	nd	nd	nd	nd	<0.0001
Salmonella sp. and *Shigella* sp.	nd	nd	nd	nd	nd	nd	nd	<0.0001
Enterococci	4.17	4.32	3.61	4.1	3.92	3.47	4.31	<0.0001
Sulphite reducing bacteria	<1	<1	<1	<1	<1	<1	<1	<0.0001

nd: Not detected, p <0.0001 indicated statistical significant difference. CFU: colony forming unit.

Table 3. Pearson correlation matrix of prevalence of microorganism's distribution.

Microorganism	Mesophil total bacteria	Yeast and mold	*S. aureus*	Total coliform	*Enterococci*	Sulphite reducing bacteria
Mesophil total bacteria	1					
Yeast and mold	-0.167	1				
Staphylococcus aureus	-0.342	0.683*	1			
Total coliform	-0.420	0.560	0.861*	1		
Enterococci	0.232	0.526	0.647*	0.584	1	
Sulphite reducing bacteria	0.235	0.741*	0.704*	0.486	0.888*	1

*Correlation is significant at the 0.05 level.

$\log_{10}$ (CFU.g^{-1}); *S. aureus* from 4.08 to 4.76 $\log_{10}$ (CFU.g^{-1}) and *Enterococci* from 3.47 to 4.31 $\log_{10}$ (CFU.g^{-1}). Total coliform was enumerated in the samples of Fada N'Gourma, Ouahigouya and Garango with ranging value of 2.14 to 2.32 $\log_{10}$ (CFU.g^{-1}). The presence of sulphite reducing bacteria was observed in all the samples analyzed. The results of microbial control of samples have shown that it did not exceed the critical limit of AFNOR (2009). The local starter is obtained after wort of sorghum fermentation. It is collected as residues in the bottom of jar and dried under the sun.

Numeration of the aerobic mesophilic bacteria on local starter samples showed microbial contamination. A similar result was determined by Atter et al. (2014) who counted 8.41 ± 0.38 $\log_{10}$ (UFC.g^{-1}) after the wort fermentation. There was a large number of aerobic mesophilic bacteria due to lack of hygienic conditions. The presence of yeast in the samples is because it is alcohol fermentation. Indeed, Lyumugabe et al. (2014) counted 8.51 $\log_{10}$ (CUF.g^{-1}) in traditional beer of Rwanda. Djegui et al. (2014), Djêgui et al. (2015), Keita et al. (2016) and N'tcha et al. (2016) showed that yeast and lactic acid bacteria were principal microorganisms in local beer starter. They numerated respectively 8.71, 9.53 and 10.35 $\log_{10}$ (UFC.g^{-1}) of yeast in ferment of Benin and Burkina Faso. The presence of mold in the samples is due to contamination caused by the spores disseminated in the environment.

Total coliform isolated in the samples of Ouahigouya, Garango and Fada N'Gourma was 2.14, 2.22 and 2.32 $\log_{10}$ (CUF.g^{-1}), respectively. Pathogenic germs such as *S. aureus*, *Enterococci* and sulphite reducing bacteria in the samples can induce food toxi-infection. Moreover, sulphite reducing bacteria regroup sporulated bacteria, including *Clostridium* which can cause food damage. *Enterococcus* sp. and *Enterococcus faecium* presence in the samples indicates fecal contamination (Dromigny, 2011).

The results are not in concordance with those of Atter et al. (2014) and Konfo et al. (2014). These did not find total coliform and *Staphylococcus* sp. in traditional beer after fermentation. This is due to the probiotic properties of yeast and lactic bacteria, according to Sawadogo et al. (2008). Moreover, traditional beer is acidic (pH 3 to 4), as mentioned by Glover et al. (2009) and Freire et al. (2014). Consequently, total coliform, *S. aureus*, sulphite reducing bacteria and *Enterococci* presence in the samples pointed to exogenous contamination due to handling during processing and drying.

Similar results of contamination of local food were observed by certain authors in others countries. Indeed, Adjrah et al. (2013) detected total coliform in the ready-to-eat salads in Lomé. Somda et al. (2014) identified *Staphylococcus* sp., *Micrococcus* sp. sulphite reducing bacteria and total coliform in *Soumbala*, a cooked condiment in Burkina Faso. Valenzuela et al. (2016) isolated *Enterococcus faecalis* and *E. faecium* in food fermented in Spain.

Correlation of prevalence of microorganisms

The results of correlation are presented in Table 3. The distribution of microorganisms in the ferments showed positive and negative correlation (p=0.005) among microbial parameters (Table 3). The highest positive correlation was observed between *Enterococci* and sulphite reducing bacteria (r=0.888). SRB also was positively correlated with yeast and mold (r=0.741) and also *S. aureus* (r=0.704). Moreover, *S. aureus* and total coliform were positive correlated (r=0.861). They were also negatively correlated with aerobic mesophil bacteria (r= -0.420).

Principal components analysis

The results of Table 4 show that analysis of principal component has demonstrated a variability of coordinate of microbial parameters found throughout the local ferment analyzed. The cumulative values of the variance of the three principal components (F1, F2 and F3) for the microbial group were 94.312%, with Eigen values ranging between 0.492 and 3.695. Principal component F1 had an Eigen value of 3.695 and contributed to 61.587% of the variation of the parameters. This principal component

Table 4. Coordinate of 7 microbial parameters and their contribution to identification of hygienic quality of the ferment.

Parameters	Principal components		
	F1	F2	F3
Mesophil aerobic bacteria	-0.077	0.785	-0.099
Yeasts and molds	0.426	-0.026	0.787
Staphylococcus aureus	0.479	-0.204	-0.145
Total coliform	0.430	-0.322	-0.430
Enterococci	0.435	0.339	-0.372
Sulphite reducing bacteria	0.457	0.351	0.161
Eigen values	3.695	1.472	0.492
Variance (%)	61.587	24.532	8.194
Cumulative (%)	61.587	86.118	94.312

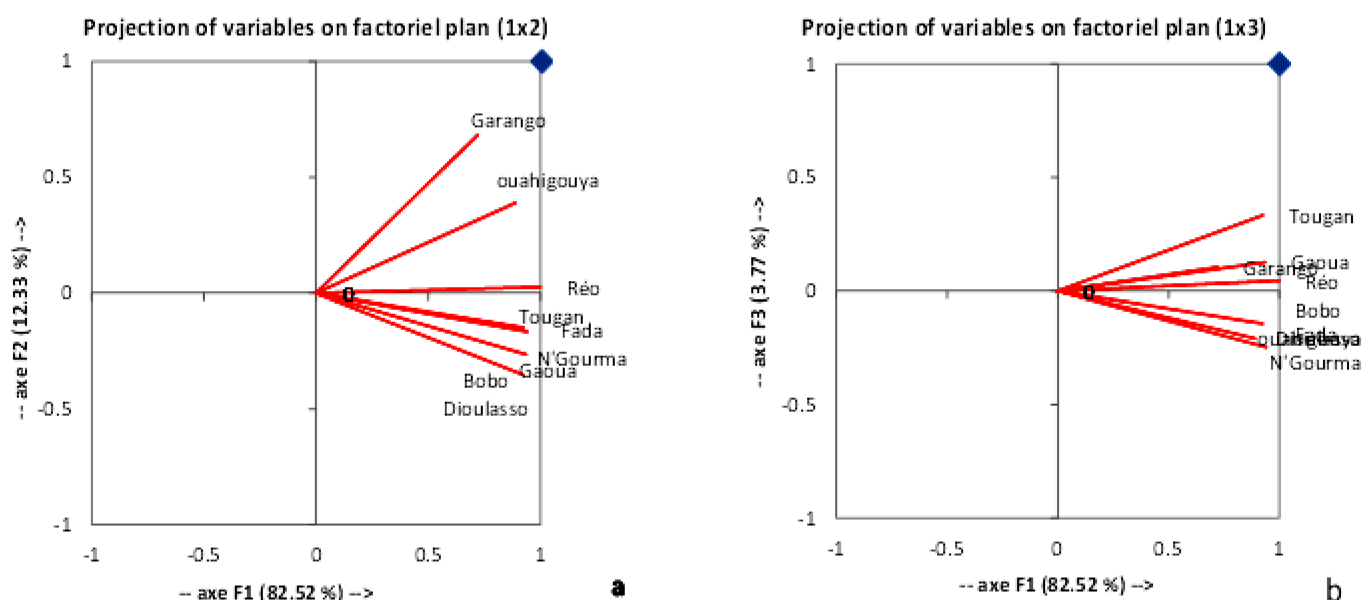

Figure 2. Distribution of locality according to ferments quality on the 1x2 and 1x3 axes of the principal components.

(F1) is associated positively to yeast and mold, *S. aureus*, total coliform, *Enterococci*, sulphite reducing bacteria. Principal components, F2 and F3 had respective Eigen values of 1.472 and 0.492; they contributed 24.532 and 8.194% to the total variation and were associated positively with rate of aerobic mesophilic bacteria, *Enterococci* and sulphite reducing bacteria.

Projection of variables on factorial plan

According to symmetrical scaling of component analysis score, the axis 1×2 and 1×3 explained respectively 94.85 and 86.29% of total inertia. The projection of variables on axis 1x2 showed a regrouping of locality according to samples. The projection plan 1x2 (Figure 2) showed a classification of 2 principal groups according to aerobic mesophilic bacteria and yeasts and molds count in the samples of the different localities. The first group (Ouahigouya, Réo and Garango) correlated positively with the two axes. The second group (Tougan, Gaoua, Bobo Dioulasso and Fada N'Gourma) correlated positively with axis 1 and negatively with axis 2.

The projection plan 1x3 showed two classifications of principal microbial group according to mesophil aerobic bacteria and *S. aureus*. The first group, regrouping (Ouahigouya, Bobo Dioulasso and Fada N'Gourma) correlated positively with axis 1 and negatively with axis 3; and a second group (Réo, Gaoua, Garango and Tougan) correlated positively with the two axes.

Conclusion

The results show that local ferment of “dolo” contained various microorganisms. The plenty number of mesophil total bacteria observed in the ferment samples could be due to lack of hygienic conditions. Pathogenic germs (*S. aureus*, total coliform, sulphite reducing bacteria, etc.) revealed that the process of ferment production need to be ameliorated for suitable and safety exploitation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Full Length Research Paper

Mosquito larvicidal trihydroxylindene derivative from submerged cultures of *Trametes* species

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Larvicidal active pure chemical compound was isolated from a *Trametes* species using conventional chemistry techniques like solvent-solvent extraction and liquid-solid adsorption techniques. Larvicidal assays carried out on the purified compound, LC_{50} and LC_{90} values were calculated and found to be 23.5 and 67.4 ppm, respectively. The chemical structures of the purified compound was elucidated using standard spectroscopic techniques: 1D (^1H; ^{13}C) and 2D (HSQC, COSY, NOESY, HMBC; DEPT) NMR experiments, assignments. The results indicate the potential of novel compounds with mosquito larvicidal activity from the *Trametes* species commonly found in the Kenyan forest ecosystems.

Key words: Mosquito larvicidal, *Aedes aegypti,* trihydroxylindene derivative, *Trametes* species.

INTRODUCTION

Mosquito-borne diseases have an economic impact, including loss in commercial and labor outputs, particularly in countries with tropical and subtropical climates; however, no part of the world (both the tropics and non-tropics) is free from vector-borne diseases (Fradin and Day, 2002). One main method for the control of mosquito-borne diseases is the use of insecticides, most of which are synthetic that affect the non-target population and also the mosquitoes are constantly developing resistance. Hence, there is a constant need for developing biologically active natural materials as larvicides, which are expected to reduce the hazards to human and other organisms by minimizing the accumulation of harmful residues in the environment. Natural products are generally preferred because of their less harmful nature to non-target organisms and due to their innate biodegradability (Rahuman et al., 2008). Bioactive compounds from nature have been the most consistent successful source for new drugs (De Silva et al., 2013).

Trametes species are group polypore fungi in the basidiomycetes division that causes wood rot and produces laccase enzymes (Isikhuemhen and Mikiashvili, 2009; Zjawiony, 2004). These are in plenty in Kenyan forests particularly during the rainy seasons and areas of high altitude like Mount Kenya forest. Whereas there are many literature reports on laccase-producing fungi, *Trametes* species is one of the best studied white-rot

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fungi, and is known to secrete several laccase isoforms (Kim et al., 2012; Montazer et al., 2009). *Trametes* species are known as some of the most efficient lignin-degrading species due to their ability to produce lignin-modifying enzymes (Baldrian, 2006). These enzymes enable degradation of lignin and a wide range of compounds with structural similarities to lignin, in addition to the reported resistance of *Trametes* species to toxic or mutagenic chemicals.

A wood rotting basidiomycete that colonized a piece of wood was collected from Mt. Kenya forest in July 2005 and immediately brought into pure culture. The fungus was serialized JO5066 and has been preserved in Integrated Biotechnology Research Laboratory (IBRL) at Egerton University as a herbarium material and pure culture. The morphological and discernable microscopic features confirmed that the fungus had clamp connections – the characteristic distinction of the basidiomycetes. The basidiomycete has been further studied using 18S RNA (ITS technique) and has been found to belong to *Trametes* species. This study clearly shows the extraction of the pure compound that was isolated from a *Trametes* species and its activity against *Aedes aegeptii.*

MATERIALS AND METHODS

Re-growth of the *Trametes* species (JO5066)

From agar slants, agar pieces with mycelia were cut from the slants and inoculated onto PDA plates under sterile conditions inside the laminar flow hood. Then the Petri dishes were sealed using the Para film and left to grow under sterile ambient laboratory conditions of 12 h light/dark cycles for 21 days.

Preparation of liquid media

Malt extract 1%, yeast 0.4% and glucose 0.4% were dissolved in 250 ml of tap water to form starter cultures. Then replicates of 1 L scale in 2 L flasks with the pH of each adjusted to 5.5. They were corked with cotton wool plugs, wrapped with aluminium foil and autoclaved at 121°C and pressure 1.5 bars for 15 min. The media was sterilized twice after which let to cool.

Inoculation of the *Trametes* species (JO5066) strain in the liquid media

A well grown pure culture of the *Trametes* species (JO5066) strain on PDA plate was cut into several agar plugs using sterile inoculating blade and in each conical flask (250 ml) four agar plugs with mycelium of the *Trametes* species (JO5066) was introduced then allowed to grow as still cultures with regular agitations at ambient conditions. The growth of the culture was then closely monitored and evaluated daily to check the biomass build up and the presence of any contamination and stopped after 7 days. A well grown 250 ml starter culture was then used to inoculate into a 1 L scale. This was allowed to grow until the glucose level in the culture was exhausted. It was done in replicates (at least fifty flasks) to ensure a high yield of the crude extracts.

Preparation of crude extracts

After growth was stopped, the culture filtrate was then separated from mycelium by filtration using a Buchner filtration system and both extracted as follows.

Culture filtrate crude extract (Kex)

The combined culture filtrate was passed through a Mitsubishi HP21-DIAION resin packed in a glass column thrice. The column was then eluted with 100% acetone, followed by 100% methanol and the eluents collected. Each organic extracts was concentrated under reduced pressure using rotary evaporator to remove acetone and methanol, respectively. The aqueous rest concentrate was extracted with equal volume of ethyl acetate thrice and the combine ethyl acetate extract dried with anhydrous sodium sulphate. The dried organic extract was concentrated using the rotary evaporator at temperatures not exceeding 50°C. The crude extract left was re-dissolved in 1 ml of methanol and kept in screw capped vials at 4°C awaiting tests and analysis.

Mycelium crude extract (Mex)

The mycelium was soaked in 3 L acetone immediately after filtration for 4 h under constant agitation using a magnetic stirrer to ensure all the compounds are extracted. It was then filtered using Buchner filtration system and the wet mycelium was dried. The dry extract was re-dissolved in 4 L water and extracted with 10 L of ethyl acetate. The combined ethyl acetate solution obtained was then dried with anhydrous sodium sulphate and concentrated using rotary evaporator. The crude extract obtained was kept in screw capped vials at 4°C awaiting tests and further analysis.

Larvicidal assay

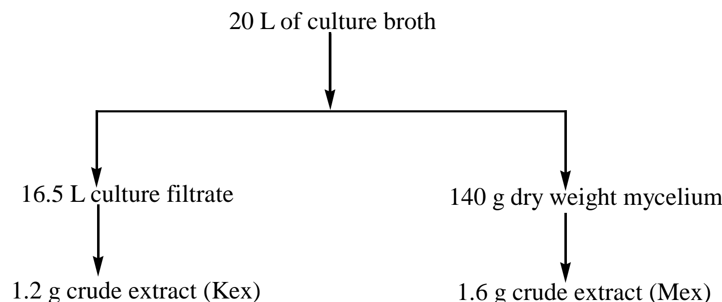
In order to establish LC_{50} and LC_{90} values (that is, the concentrations of extracts in parts per million required to kill 50 and 90% of *Aedes aegypti* larvae, within 24 h), multiple 10-fold dilutions of the extract stock solution was prepared to provide a working concentration range. Two replicate assays were carried out for every sample concentration, each with 10 larvae. Larvae were observed at the start of the assay, 2, 4, and 8 h interval and after 24 h and considered dead when they did not respond to stimulus or when they did not rise to the surface of the solution. Negative controls accompanied each assay and involved treating larvae with water and methanol. The LC_{50} and LC_{90} were calculated only for the most active extracts. The dead larvae counted after every 2 h, was used to calculate the percentage mortality reported from the average for the three replicates taken.

$$\% Mortality = \frac{Number of\ dead larvae}{Number of\ Larave Introduced} x100$$

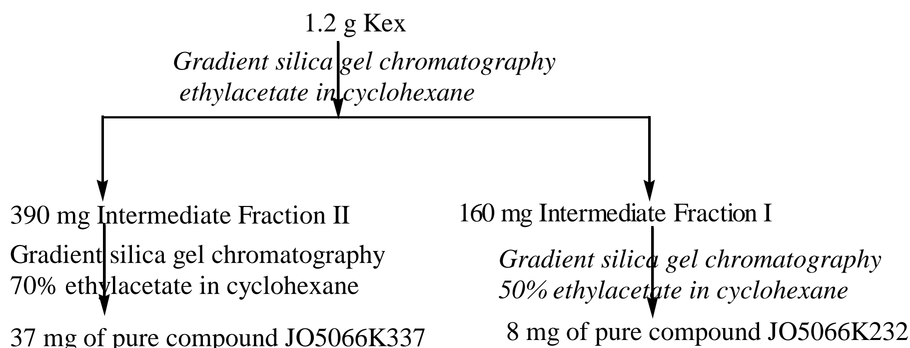
RESULTS AND DISCUSSION

Cultivation and purification of the basidiomycete, *Trametes* (JO5066)

The basidiomycete JO5066 was cultivated as still cultures under laboratory ambient conditions and the growth was stopped after 21 days when the glucose



Scheme 1. Extraction.



Scheme 2. Purification of intermediate fractions.

levels were depleted. Immediately growth was stopped, crude extracts were prepared for both intra- and extra-cellular secondary metabolites as summarized in Scheme 1. The crude extract from culture filtrate (Kex) was 1.2 g while the mycelium crude extract (Mex) was 1.6 g.

The crude extracts were tested for larvicidal activity and Kex was found to have activity for as low as 50 ppm concentration within the range 30 to 50% mortality for the whole period of mortality. It was also observed that 100% mortality occurred for concentrations from 500 ppm except for 200 ppm after 8 h. While for Mex, activity was observed from 500 ppm in the range 30 to 70% for the whole period the experiment was evaluated. It is only at 1000 ppm that 100% mortality was observed.

Bioactivity activity guided purification of compounds from Kex

The biologically active compounds in culture filtrate (Kex) were targeted for purification using bioactivity guided purification. This was done using silica gel chromate-graphy. The crude extract was purified according to the scheme summarized in Scheme 2.

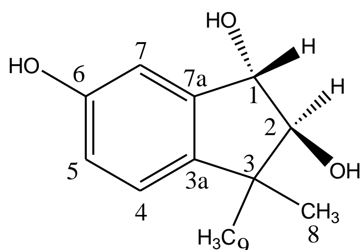
The 1.2 g yield of the crude extract (Kex) was subjected to chromatography with gradient elution with increasing polarity of ethyl acetate in cyclohexane. The active compounds eluted with gradient mobile phase (cyclohexane and ethylacetate) to afford 160 mg of an intermediate FI and 390 mg fraction labelled II respectively. These intermediate fractions were further subjected to chromatographic separation, which led to elution of 8 and 37 mg of pure compounds JO5066K232 with 50% ethyl acetate in cyclohexane and JO5066K337 with 70% ethyl acetate in cyclohexane respectively.

Mosquito larvicidal activity of the pure compounds

The purified compound, JO5066K337 (2,3-dihydro-3,3-dimethyl-1H-indene-1,2,6-triol), was tested for larvicidal activity against *A. aegypti*. There was no observable activity up to 8 h since the start of the experiment. From the results, the mortality data at 24 h was correlated to obtain LC_{50} = 235 ppm and LC_{90} = 674 ppm.

The pure compound JO5066K337 was obtained as a yellow liquid weighing 37 mg. The chemical structure of the compound was determined based on 1D and 2D NMR experiments. From this the structure of compound JO5066K337 was discerned and proposed to be 2, 3-dihydro-3, 3-dimethyl-1H-indene-1, 2, 6-triol. This is a new compound being reported for the first time from cultures of a *Trametes* species. From ^{13}C-NMR spectrum there were 11 discernable carbon signals, which when

analyzed together with ^{1}H-NMR and DEPT spectra indicated that there were 4 quaternary carbons, 5 methine carbons and 2 methyl carbons. It was evident that the compound had no methylene carbons. The information was found to be consistent with the molecular formula, $C_{11}H_{14}O_3$, with unsaturation index (UI) of 5.



2, 3-dihydro-3, 3-dimethyl-1H-indene-1, 2, 6-triol

From the 11 signals there were 6 typical aromatic sp^2 hybridized carbons; 111.8, 124.3, 129.3, 132.8, 135.7 and 168.3ppm, with the last one (C-6) being oxygenated typified by the chemical shift in the deshielded range and consistent with a phenol moiety. From these carbons C-3a (124.3 ppm) and C-7a (132.8ppm) are quaternary and C-4 (135.7 ppm), C-5 (129.3 ppm) and C-7 (111.8 ppm) are bearing a proton each.

The remaining 5 carbons were accounted for by 2 methyl carbons, 11.4 ppm (C-8) and 16.8 ppm (C-9), with both attached to a quaternary carbon 22.1 ppm (C-3). This leaves 2 carbons that are accounted for as oxygenated sp^3 hybridized that are part of a rigid ring system, C-1 (83.3 ppm) and C-2 (78.9 ppm). Carbons C-1, C-2 and C-3 are part of five-membered ring that is fused to the aromatic system as deciphered from the 2-D NMR experiments COSY, NOESY and HMBC. The two protons (4.72 and 3.72 ppm) attached to C-1 and C-2, respectively are in a *trans*- orientation supported by a ^3J-value of 2.1 Hz characteristic of such a stereochemistry common in sugars (Friebolin, 2005). It is on this basis that the relative configuration on C-1 and C-2 were proposed.

Conclusion

The research findings in this work revealed that the basidiomycete, *Trametes* sp. (JO5066) produced biologically active compounds when grown in liquid submerged cultures for 21 days. The crude extract from culture filtrate showed both larvicidal activity against *A. aegyptii* and antibacterial and antifungal activities. The crude extract showed 100% mortality of the mosquito larvae for a concentration of 50 ppm within 24 h. These observations clearly demonstrated that the *Trametes* sp (JO5066) was producing biologically active compounds.

The crude extracts when fractionated and purified for the active compounds responsible for the observed biological activities afforded the compound 2, 3-dihydro-3, 3-dimethyl-1H-indene-1, 2, 6-triol whose structure was elucidated based on NMR experiments. 2, 3-Dihydro-3, 3-dimethyl-1H-indene-1, 2, 6-triol showed clear larvicidal activity while it had weak and insignificant antimicrobial activities. The compound had a considerable activity with LC_{50} of 23.5 ppm and LC_{90} of 67.4 ppm against the third instar larvae of *A. aegyptii*. This is a major finding for this study given that the compound is a new compound reported for the first time from cultures of a fungal source.

CONFLICT OF INTERESTS

The authors confirm that there is no conflict of interests. The funding agencies had no role in the review framework, concepts, interpretation of literature and the final conclusions. The conceptualization, design of the research, the execution of the research methods, collection, analysis and interpretation of data leading to the derived conclusions is wholly our responsibility as a research team.

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Full Length Research Paper

Traditional dishes consumed in Zimbabwe and high-performance liquid chromatography (HPLC) quantitation of their antioxidant phytochemicals

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Diabetes type 2, cancers, hypertension and obesity have become problematic diseases worldwide calling for more studies on their prevention and management. Antioxidant phytochemicals have shown potential to reduce cases of such chronic diseases therefore the present study quantified antioxidant phytochemicals in traditional dishes consumed in Zimbabwe. Traditional dishes are readily available therefore are cheap and accessible sources of food. Antioxidant phytochemicals were extracted using matrix solid phase dispersion and thin layer chromatography coupled to 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and then quantified using high performance liquid chromatography. The levels of antioxidant phytochemicals ranged from 2 ± 0.5 to 620 ± 3 mg/g for the ethanol extract and 20 ± 3 to 377 ± 5 mg/g for the ethyl acetate extract. Type and amounts of antioxidant phytochemical present in a dish depended upon the thick porridge and relish combination selected. Combinations involving wild vegetables as relish consisted of better levels of antioxidant phytochemicals than meat combinations. No dish consisted of all the types of antioxidant phytochemicals therefore it is necessary to eat a variety.

Key words: Diabetes type 2, cancers, hypertension, obesity, phytocompounds.

INTRODUCTION

Chronic diseases such as diabetes type 2, obesity, hypertension, and cancers have become a burden to the general public and governments worldwide. Specific lifestyle and dietary practices have shown either to decrease or increase the risk of chronic diseases (WHO, 2005). People have undergone significant changes in lifestyle and dietary practices whereby a decline in physical activity and consumption of foods consisting of high levels of carbohydrates and fats has taken a center stage (AFN, 2007). Increased consumption of trans-fatty acids, carbohydrates and sodium increases the risk of obesity and cardiovascular diseases (Soriano Garcia,

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2013). Diabetes type 2 is increased by taking saturated fats and carbohydrates (Stadler, 2012). Therefore efforts to mitigate chronic diseases should mainly target the diet.

Although sticking to a healthy diet is an important aspect in prevention of chronic diseases however it requires its availability and affordability. Poverty results in people taking foods that promote chronic diseases. Families with small incomes tend to buy more processed and unhealthy foods in large retail grocery shops because the prizes are affordable (Gordon and Oddo, 2012; Mullany et al., 2013). Therefore interventions to be made should consider availability and affordability of healthy foods in the community. Traditional foods offer the best option since they are locally available. Traditional diets previously played an important role in food security and prevention of chronic diseases which were very rare in most African societies but now are on an increasing trend. It is believed that the increase in cases of chronic diseases is as a result of a decrease in the consumption of traditional foods (Jones Smith et al., 2013). Previous studies show a possibility of mitigating chronic diseases that directly affect human kind by improving dietary intake of antioxidant phytochemicals (Wang et al., 2013; Zhang et al., 2016).

The term antioxidant phytochemicals refers to chemical compounds which occur naturally in plants. They are responsible for their attractive colours such as, purple and red colours and typical smells such as those of herbs and fruits. Antioxidant phytochemicals that have been reported previously are mainly, phenolic acids and polyphenols (Andersen and Markham, 2006; Oh et al., 2011). Examples include sinapic acid, caffeic acid, chlorogenic acid, gallic acid, quercetin, kaempferol, rutin and catechin (Shetty et al., 2013; Naczk and Shahidi, 2004).

Antioxidant phytochemicals reduce the dangerous effects of reactive oxygen species by neutralizing them. Reactive oxygen species, produced by normal metabolic reactions or from the environment have been shown to be the major causes of cancers (Lushchak, 2011; Kyro et al., 2013), diabetes type 2 (Pitocco et al., 2013; Mursu et al., 2014), hypertension (Banday et al., 2007) and cardiovascular diseases (Yamada et al., 2011). Past studies have shown that raw traditional fruits and vegetables consist of significant amounts of antioxidant phytochemicals (Muchuweti et al., 2009; Chipurura et al., 2013; Carlsen et al., 2010; Mandindi, 2015), however little is known about the complete profile of antioxidant phytochemicals in traditional dishes especially those consumed in Africa (Mavengahama et al., 2013). In Zimbabwe and most African countries traditional dishes mainly consist of small grains or maize mealie meal thickened porridge (sadza, tsima, itschwala, phutu, or ugali) which is eaten in combination with a variety of boiled wild vegetables and seeds or meat as relish. This study therefore was designed to profile the different traditional dishes and quantify their antioxidant phytochemicals.

MATERIALS AND METHODS

Chemicals

Chemical standards, salicylic acid, gallic acid, chlorogenic acid, caffeic acid, vanillic acid, p-coumaric acid, ferulic acid, quercetin, kaempferol, rutin, HPLC grade solvent (methanol), DPPH (2, 2-Diphenyl-1-picrylhydrazyl), hydrophilic lipophilic balance (HLB) water wettable sorbent and TLC plates, silica gel $60F_{254}$ (20 × 20 cm) and silica gel on aluminium strips (1.5 × 10 cm) were purchased from Sigma chemical company (St Louis MO, USA). All other chemicals were of analytical grade and were purchased from SkyLabs, Springfield, Johannesburg, South Africa.

Sample collection

Traditional dishes were purchased in triplicate from food outlets around the city of Harare and from rural areas in Mashonaland East and Central. They were directly transported in a cooler box with ice to the laboratory. Some were prepared by the authors under the supervision of an experienced elderly person using raw materials bought from Mbare musika, a national market place in Harare, Zimbabwe. Samples were kept under refrigeration in the dark until required for analysis. Samples were selected basing on availability and affordability. Description of samples is shown in Table 1. Two “affluent” dish types, polished maize seed thick porridge and beef stew and fresh chips and broiler chicken from popular fast food outlets were also purchased in triplicate for comparison with the traditional dishes.

Sample preparation

Matrix solid phase dispersion was used as an initial method for extracting phytochemicals from the blended samples (Barker, 2007). HLB (0.04 g) was thoroughly mixed with 2 g of the blended and homogenized sample using a mortar and pestle. The resultant mixture was then loaded into 10 mL fritted propylene syringe barrels. Packed barrels were washed with ultrapure water prepared internally using Millipore water purification unit and vacuum dried for 2 h. Elution of phytochemicals was achieved using 12 mL of ethanol under vacuum to speed up the process. The eluent was then vacuum dried on a rotary evaporator at 60°C. The contents were weighed and redissolved in 1 mL of ethanol and stored in a refrigerator until required for thin liquid chromatographic (TLC) analysis. Elution was repeated twice to ensure that all the compounds of interest were extracted from the matrix and sorbent. The process was repeated for the ethyl acetate extract.

Analytical TLC analysis

Aluminium silica gel coated strips (1.5 × 10 cm) were activated by heating gentle in an oven and then each spotted with standard premix, ethanol and ethyl acetate extracts. Spots were visualized by spraying revealing agents and observing under UV lamp at 254 and 375 nm using a Spectroline UV viewing cabinet. Several solvent mixtures were tested in the separation of the standard premix consisting of caffeic, chlorogenic, gallic, salicylic and vanillic acid, quercetin and kaempferol in order to determine the best solvent mixture that would efficiently separate the phenolics. The best solvent mixture was found to be ethanol/acetic acid/water in the ratio 6:11:3 (v/v) (Figure 1) and this was used throughout the

Table 1. Samples collected or prepared for analysis.

Shona or common name	English or botanical name
Sadza remhunga + road runner	Pearl millet thick porridge + traditional chicken stew
Sadza remhunga + nyama yemombe	Pearl millet thick porridge + beef stew
Sadza remhunga + muchacha	Pearl millet thick porridge + *Cucumis anguria* leaves
Sadza remhunga + mowa	Pearl millet thick porridge + *Amaranthus spinosus* leaves
Sadza remhunga + nyevhe	Pearl millet thick porridge + *Cleome gynandra* leaves
Sadza remhunga + derere rebupwe	Pearl millet thick porridge + *Cratotheca spp* leaves
Sadza remhunga + muboora	Pearl millet thick porridge + *Cucurbita maxima* leaves
Sadza remhunga + tsine	Pearl millet thick porridge + *Bidens pilosa* leaves
Sadza remhunga + mukaka wakakora	Pearl millet thick porridge + sour milk
Sadza rezviyo + road runner	Finger millet thick porridge + traditional chicken stew
Sadza rezviyo + beef stew	Finger millet thick porridge + beef stew
Sadza rezviyo + muchacha	Finger millet thick porridge + *C. anguria* leaves
Sadza rezviyo + mowa	Finger millet thick porridge + *A. spinosus* leaves
Sadza rezviyo + nyevhe	Finger millet thick porridge + *C. gynandra* leaves
Sadza rezviyo + derere rebupwe	Finger millet thick porridge + *C. spp* leaves
Sadza rezviyo + muboora	Finger millet thick porridge + *C. maxima* leaves
Sadza rezviyo + tsine	Finger millet thick porridge + *B. pilosa* leaves
Sadza rezviyo + mukaka wakakora	Finger millet thick porridge + sour milk
Sadza remapfunde + road runner	Sorghum thick porridge + traditional chicken stew
Sadza remapfunde + mowa	Sorghum thick porridge + *A. spinosus* leaves
Sadza remupunga + road runner	Rice thick porridge + traditional chicken stew
Sadza remupunga + munyemba	Rice thick porridge + *Vigna unguiculata* leaves
Sadza remugaiwa + road runner	Unpolished *Zea mays* thick porridge + traditional chicken stew
Sadza remugaiwa + nyama yemombe	Unpolished *Z. mays* thick porridge + beef stew
Sadza remugaiwa + muchacha	Unpolished *Z. mays* thick porridge + *Cucurbita maxima* leaves
Sadza remugaiwa + mowa	Unpolished *Z. mays* thick porridge + *Amaranthus spinosus* leaves
Sadza rakasvuurwa + nyevhe	Polished *Z. mays* thick porridge *Cleome gynandra* leaves
Sadza rakasvuurwa + beef stew	Polished *Z. mays* thick porridge + beef stew
Sadza rakasvuurwa + chicken stew	Polished *Z. mays* thick porridge + broiler chicken stew
Fried Irish potato chips and chicken	Fried Irish potato chips and broiler chicken

study in separating sample extracts. Elution was allowed through a distance of 8 cm in an air tight development chamber. The developed plates were air dried and then kept in a cool and dry place until required for phytochemical screening.

Analysis of phenolic compounds

The developed chromatographic strips were dried briefly over a hot plate and then sprayed with Folin-Ciocalteu reagent and ethanolic aluminium chloride (Singh et al., 2012).

Preparative TLC analysis

Silica gel TLC plates ($60F_{254}$, 20 × 20 cm) were activated and marked at each edge and then the extracts were applied in the form of bands of 2 mm in height. The plates were developed over a distance of 12 cm using a solvent mixture consisting of ethanol, acetic acid and water in the ratio 6:11:3 (v/v). The plates were then air dried at room temperature. The bands were scratched into test tubes and dissolved in 2 mL of original solvent. The contents were vacuum filtered using Whatman no. 1 filter paper followed by 0.45 µm glass sample filters. The contents were placed in amber glass vails until required for antioxidant, UV and HPLC analysis.

Determination of antioxidant activity

All the solutions from the scratched bands were analyzed for antioxidant activity. This was performed by adding 5 drops of extract into 1 mL of 0.01 M ethanolic solution of DPPH. The time the colour changed from purple to yellow and colour intensities were noted (Figure 3). The bands which showed very strong antioxidant activity were further analyzed using HPLC to quantify the phytochemicals.

HPLC analysis

Analysis of standards and samples were performed on a Varian HPLC coupled to a prostar 325 variable UV detector. The detector was controlled remotely by the Varian star or Galaxie workstation version 6 software. Samples were separated on a Varian Microsorb MV 1005 packed C_{18} column, 250 × 4.6 mm id and 5 µm particle size. An isocratic separation mode was used. Different mobile

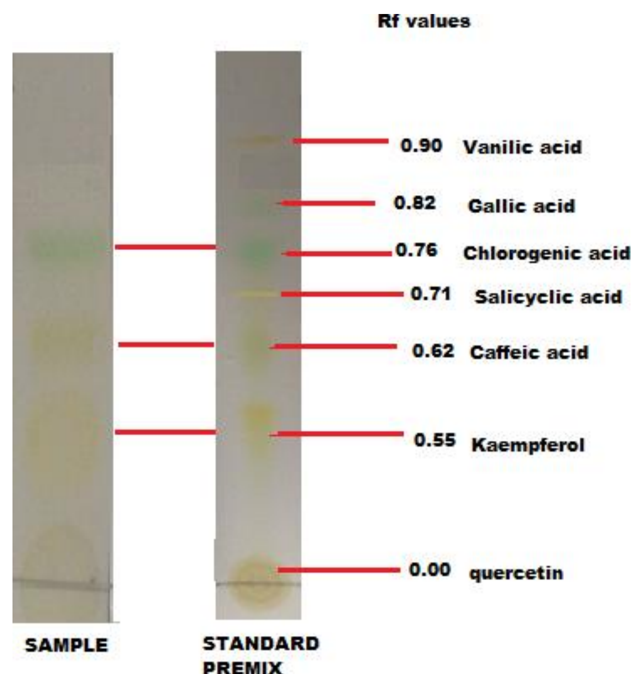

Figure 1. Typical analytical TLC separation chromatograms for sample extract and standard premix.

phases were tested in order to determine the mobile phase that would best separate the standards efficiently. The most efficient mobile phase consisted of HPLC grade methanol and 1% aqueous acetic acid prepared in the ratio 25:75 (v/v). The mobile phase was prepared every day and degassed in a sonicator at 30°C for 5 min. Analysis conditions were room temperature, flow rate 1 mL/min and injection volume, 20 µL. The wavelength of analysis was 310 nm (Gird et al., 2014) which was also confirmed by scanning samples and standard premixes over the range of 200 to 1000 nm on a UV spectrophotometer. Standard premix was prepared by mixing 1000 mg/mL of each standard, caffeic, chlorogenic, gallic, salicylic and vanillic acid (phenolic acids) and quercetin and kaempferol (flavonoids). The calibration curve was determined over a concentration range of 1 to1000 mg/mL and was used for quantification. Peak identification was performed by comparing pick retention times and by spiking individual standards into selected samples. Limit of detection and quantification was found to be 0.01 and 0.08 ng/mL. The percentage recovery obtained by spiking 2 mg/mL of selected standards in sample matrix ranged from 89.3 ± 0.6 to 92.5 ± 0.6%.

RESULTS

Traditional dish samples

A total of 27 different traditional dishes (Table 1) were encountered during sampling. Most of them consisted of small grains thick porridge and wild vegetables leaves or meat as relish. The relish were prepared by either boiling or frying in cooking oil without addition of spices. Three other dishes consisting of polished maize meal thick porridge and fried chips were collected for comparison sake. These dishes are considered to be modern and are consumed more often by most affluent families in Zimbabwe.

Analytical TLC

R_f values for the analytical TLC analysis of the several dishes are shown in Table 2. The R_f values ranged from 0.05 to 0.96. The ethyl acetate extract produced more spots than the ethanol extract.

Analysis of phenolic compounds

Analyses to test for presence of phenolic compounds showed that most spots for traditional dishes tested positive for presence of phenolic compounds while all spots for polished *Zea mays* seeds and meat tested negative. Unpolished *Zea mays* seeds and meat dishes tested positive for phenolic compounds. Comparing R_f values for samples and those for standard premix (Figure 1) shows that the traditional dishes consist of phytochemicals with the same R_f values as the standards, caffeic, chlorogenic, gallic, salicylic and vanillic acid and quercetin and kaempferol. Therefore these standards were used for HPLC quantitative analysis of antioxidant phytochemicals exhibiting very strong antioxidant activity.

Antioxidant activity analysis

Separation of the phenolics in the samples was achieved using preparative TLC plates visualized by spraying

Table 2. Analytical TLC and phytochemical analysis.

Dish name	Ethanol extract		Ethyl acetate extract	
	R_f values	Phenolic test	R_f values	Phenolic test
Pearl millet thick porridge + traditional chicken stew	0.50	+	0.06 0.90	+ +
Pearl millet thick porridge + beef stew	0.50	+	0.05	+
Pearl millet thick porridge + *Cucumis anguria* leaves	0.50 0.70	- +	0.05 0.12 0.90	- + +
Pearl millet thick porridge + *A. spinosus* leaves	0.50 0.11	+ +	0.06 0.14	- +
Pearl millet thick porridge + *Cleome gynandra* leaves	0.50 0.70	+ +	0.05 0.13 0.84	- + +
Pearl millet thick porridge + *Cratotheca* spp. leaves	0.50 0.82	- +	0.06 0.09 0,10	- - +
Pearl millet thick porridge + *C. maxima* leaves	0.50 0.94	+ +	0.06 0.09 0.53	- + +
Pearl millet thick porridge + *B. pilosa* leaves	0.50 0.68	+ +	0.05 0.76	+ +
Pearl millet thick porridge + sour milk	0.50	+	0.06 0.10	- +
Finger millet thick porridge + traditional chicken stew	0.71 0.85	+ +	0.09 0.92	+ +
Finger millet thick porridge + beef stew	0.71	+	0.08	+
Finger millet thick porridge + *C. anguria* leaves	0.71 0.88	+ +	0.14 0.84 0.94	+ + +
Finger millet thick porridge + *A. spinosus* leaves	0.71 0.83	+ +	0.12 0.77 0.85	+ + +
Finger millet thick porridge + *C. gynandra* leaves	0.72 0.88	+ +	0.32 0.55 0.76	+ + +
Finger millet thick porridge + *Cratotheca* spp. leaves	0.72 0.89	+ +	0.13 0.77 0.92	+ + +
Finger millet thick porridge + *C. maxima* leaves	0.73 0.90	+ +	0.15 0.79 0.90	+ + +
Finger millet thick porridge + *B. pilosa* leaves	0.71 0.86	+ +	0.13 0.80 0.98	+ + +

Table 2. Contd.

Finger millet thick porridge + sour milk	0.72 0.88	+ +	0.12 0.81	+ +
Sorghum thick porridge + traditional chicken stew	0.42 0.59	+ +	0.09 0.66	+ +
Sorghum thick porridge + *A. spinosus* leaves	0.42 0.79	+ +	0.17 0.64 0.81	+ + +
Rice thick porridge + traditional chicken stew	0.12 0.60	+ +	0.05 0.61	+ +
Rice thick porridge + *V. unguiculata* leaves	0.55 0.78	+ +	0.15 0.69	+ +
Unpolished *Z. mays* thick porridge traditional + chicken stew	0.15 0.30	- +	0.07 0.42	- +
Unpolished *Z. mays* thick porridge + beef stew	0.16	+	0.09	-
Unpolished *Z. mays* thick porridge + *Cucurbita maxima* leaves	0.13 0.77	+ +	0.09 0.83	+ +
Unpolished *Z. mays* thick porridge + *Amaranthus spinosus* leaves	0.17 0.82	+ +	0.09 0.90	+ +
Polished *Z. mays* thick porridge *Cleome gynandra* leaves	0.12 0.67	- +	0.12 0.96	- +
Polished *Z. mays* thick porridge + beef stew	0.11	-	0.05	-
Polished *Z. mays* thick porridge + broiler chicken stew	0.11	-	0.09	-
Fried Irish potato chips and broiler chicken	0.80 0.83 0.95	- - -	0.11 0.53	- -

-, Negative; +, positive.

revealing agents and a UV lamp at 254 and 375 nm. Extracts were applied on the TLC plates in the form of bands (Figure 2). Results for antioxidant analysis using DPPH a widely used radical for testing antiradical activity are shown in Table 3. Antioxidant activity was ascertained by a change of colour of the DPPH solution from purple to yellow (Figure 3). The results show that most traditional dishes exhibited antioxidant activity. Antioxidant activity ranged from moderate to very strong. Bands exhibiting very strong antioxidant activity were scratched and amounts of antioxidant phytochemicals quantified using HPLC. All bands for unpolished *Zea mays* seeds and meat dishes did not show antioxidant activity.

HPLC analysis

Standard calibration curves were generated by plotting peak area against concentration. Standard calibration curves for all other standards except salicylic acid showed good linearity between 1 and 1000 mg. Salicylic acid showed good linearity between 1 and 800 mg (Table 4). The calibration curves were used for quantification of antioxidant phytochemicals in traditional dishes. Typical chromatograms observed for HPLC analysis of standard premix and extracts are shown in Figure 4 The concentration of antioxidant phytochemicals are shown in Tables 5 and 6. Ethanol extract showed more bands with very strong antioxidant activity than ethyl acetate extracts however ethyl acetate extract had more bands than the ethanol extract. Concentration of antioxidant phytochemicals in the ethanolic extract ranged from 2 ± 0.5 to 620 ± 3 while that for ethyl acetate ranged from 20 ± 3 to 377 ± 5 mg/g sample. The phytochemical with the highest concentration was salicylic acid in finger millet thick porridge and *C. anguria* boiled leaves at R_f value of 0.71 for ethanolic acid and chlorogenic acid in pearl millet thick porridge and *B. pilosa* leaves at R_f value of 0.76 in ethyl acetate extract.

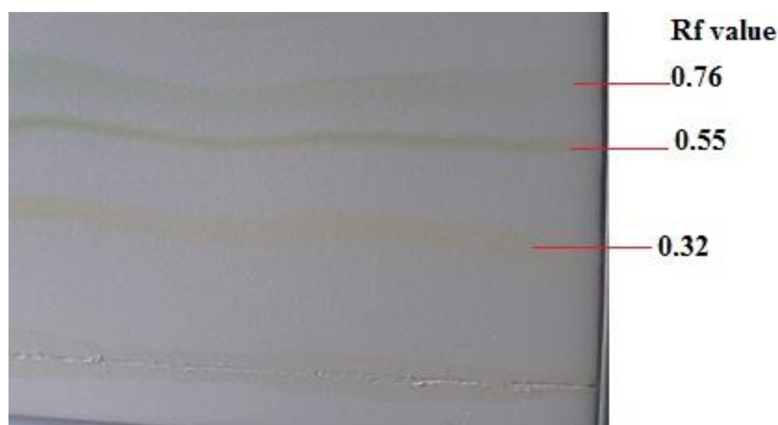

Figure 2. Preparative TLC separation of phenolic compounds in ethyl acetate extract.

Table 3. DPPH antiradical activity analysis.

Dish name	Ethanol extract		Ethyl acetate extract	
	R_f values	DPPH test	R_f values	DPPH test
Pearl millet thick porridge + traditional chicken stew	0.50	+++	0.06 0.90	+ ++
Pearl millet thick porridge + beef stew	0.50	++	0.93	+
Pearl millet thick porridge + *C. anguria* leaves	0.50 0.70	- +++	0.05 0.12 0.90	- + ++
Pearl millet thick porridge + *A. spinosus* leaves	0.11 0.51	+ +++	0.06 0.14	- +++
Pearl millet thick porridge + *C. gynandra* leaves	0.50 0.70	+++ +++	0.05 0.13 0.84	- ++ ++
Pearl millet thick porridge + *Cratotheca* spp. leaves	0.50 0.82	- +++	0.06 0.09 0,10	- - ++
Pearl millet thick porridge + *C. maxima* leaves	0.50 0.94	+ +++	0.06 0.09 0.53	- ++ +++
Pearl millet thick porridge + *B. pilosa* leaves	0.50 0.68	+++ +++	0.05 0.76	+ +++
Pearl millet thick porridge + sour milk	0.50	++	0.06 0.10	- ++
Finger millet thick porridge + traditional chicken stew	0.71 0.85	+ ++	0.09 0.92	+ ++
Finger millet thick porridge + beef stew	0.71	+	0.08	+
Finger millet thick porridge + *C. anguria* leaves	0.71 0.88	+++ ++	0.14 0.84 0.94	++ ++ +++

Table 3. Contd.

Finger millet thick porridge + *A. spinosus* leaves	0.71 0.83	++ +++	0.12 0.77 0.85	+ ++ +++
Finger millet thick porridge + *C. gynandra* leaves	0.72 0.88	+++ +++	0.32 0.55 0.76	+ ++ +++
Finger millet thick porridge + *C.* spp. leaves	0.72 0.89	+ +++	0.13 0.77 0.92	+ ++ ++
Finger millet thick porridge + *C. maxima* leaves	0.73 0.90	+ +++	0.15 0.79 0.90	+ ++ +++
Finger millet thick porridge + *B. pilosa* leaves	0.71 0.86	++ +++	0.13 0.80 0.98	+ +++ ++
Finger millet thick porridge + sour milk	0.72 0.88	+ ++	0.12 0.81	+ ++
Sorghum thick porridge + traditional chicken stew	0.42 0.59	+ +++	0.09 0.66	+ ++
Sorghum thick porridge + *A. spinosus* leaves	0.42 0.79	+ +++	0.17 0.64 0.81	+ + ++
Rice thick porridge + traditional chicken stew	0.12 0.60	+ ++	0.05 0.61	+ ++
Rice thick porridge + *V. unguiculata* leaves	0.55 0.78	+ +++	0.15 0.69	+ +++
Unpolished *Z. mays* thick porridge traditional + chicken stew	0.15 0.30	- +	0.07 0.42	- ++
Unpolished *Z. mays* thick porridge + beef stew	0.16	-	0.09	-
Unpolished *Z. mays* thick porridge + *C. maxima* leaves	0.13 0.77	+ +++	0.09 0.83	+ +++
Unpolished *Z. mays* thick porridge + *A. spinosus* leaves	0.17 0.82	+ +++	0.09 0.90	+ ++
Polished *Z. mays* thick porridge *C. gynandra* leaves	0.12 0.67	- +++	0.12 0.96	- +++
Polished *Z. mays* thick porridge + beef stew	0.11	-	0.05	-
Polished *Z. mays* thick porridge + broiler chicken stew	0.11	-	0.09	-
Fried Irish potato chips and broiler chicken	0.80 0.83 0.95	- - -	0.11 0.53	- -

-, no; +, moderate; ++, strong; +++, very strong antioxidant activity.

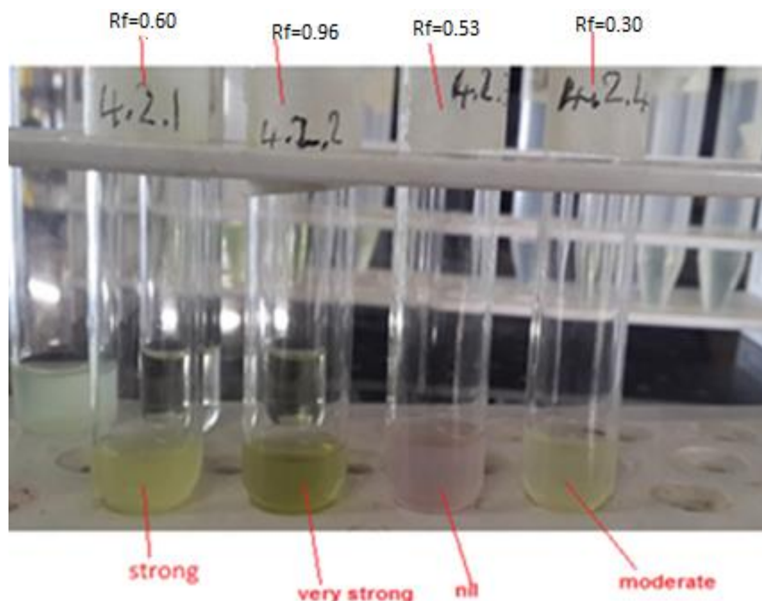

Figure 3. DPPH antiradical activity.

Table 4. Standard calibration curves.

Standard phytocompound	Linear dynamic range/mg	Equation	R^2
Salicylic acid	1-800	Y=15447x-90845	0.9986
Gallic acid	1-1000	Y=9145.2x+70346	0.9991
Chlorogenic acid	1-1000	Y=11345x+222100	0.9982
Caffeic acid	1-1000	Y=11859x+231200	0.9946
Vanillic acid	1-1000	Y=13199x-115613	0.9965
Quercetin	1-1000	Y=380.37x-772.83	0.9955
Kempferol	1-1000	Y=5834.6x+10453	0.9999

DISCUSSION

Ethanol, acetic acid and water in the ratio 6:11:3 (v/v) was found to be the best mobile phase that could separate the phytochemicals efficiently. Ethanol extracts gave 1 to 2 spots while ethyl acetate extract gave 1 to 3 spots depending on the type of the dish. The polarity of solvent has been previously observed to contribute to the type of phenolic compound that can be extracted. Flavones and less polar phenolic acids have been previously extracted using ethyl acetate and chloroform (Naczk and Shahidi, 2004). Alcohols or alcohol-water mixtures were found to be the best solvents for extracting flavonoid glycosides and more polar phenolic acids (Naczk and Shahidi, 2004).

Plant phenolics are responsible for antioxidant activity of plant extracts (Adedapo et al., 2011). Most traditional dishes studied in the present study consist of phenolics and antioxidant activity however in different amounts. Polished maize thick porridge and meat as relish tested negative for both phenolics and antioxidant tests (Tables 2 and 3). This shows that phenolic compounds in the traditional dishes may be responsible for the observed antioxidant activity. This was further substantiated by the results observed for analytical TLC analysis of extracts and standard premix (Figure 1). Based on the present results, each traditional dish at least consists of either a phenolic acid or a flavonoid exhibiting a very strong antioxidant activity. The contents of phenolics portraying a very strong antioxidant activity varied depending on dish combinations. For instants, pearl millet thick porridge and traditional chicken stew (R_f value 0.50) consist of quercetin and kaempferol only while pearl millet thick porridge and *C. anguria* leaves (R_f value 0.70) consist of salicylic acid and caffeic acid (Table 5). It is important to note that combining thick porridge with vegetables offer better results than meat products. Polished *Z. mays* thick porridge with boiled *Cleome gynandra* leaves as relish portrayed strong antioxidant activity while polished *Z. mays* thick porridge with either broiler chicken or beef stew exhibited no antioxidant activity. Pearl millet thick porridge and traditional chicken stew exhibited very

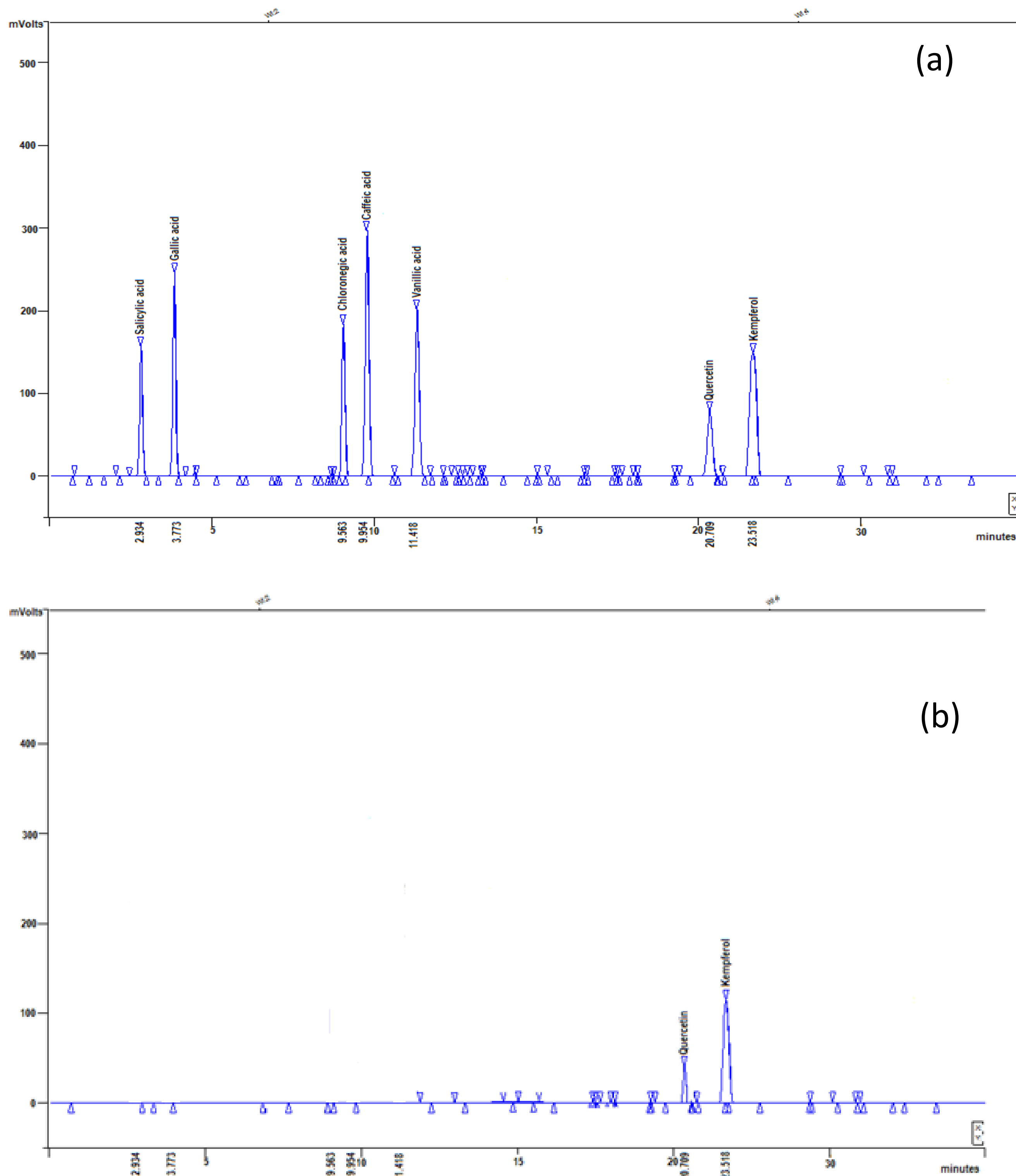

Figure 4. Typical HPLC chromatograms for (a) standard premix (b) sample.

strong antioxidant activity than pearl millet thick porridge and beef meat. The antioxidant activity of the former may be attributed to the traditional chicken which is raised using small grains and wild vegetables as its food. Antioxidant activity depends on the number of hydroxyl groups present in the molecule. It is believed that the

Table 5. Concentration of antioxidant phytochemicals in ethanol extract.

Dish name	Concentration of antioxidant phytochemicals (mg/g of sample ± SD)						
	Salicylic acid	Gallic acid	Caffeic acid	Chlorogenic acid	Vanillic acid	Quercetin	Kempferol
Pearl millet thick porridge + traditional chicken stew; Rf value 0.50						69 ± 2	139 ± 1
Pearl millet thick porridge + *C. anguria* leaves Rf value 0.70	256 ± 5		37 ± 2				
Pearl millet thick porridge + *A. spinosus* leaves Rf value 0.51						2 ± 0.5	155 ± 2
Pearl millet thick porridge + *C. gynandra* leaves Rf value 0.50						3 ± 1	370 ± 2
Rf value 0.70	507 ± 3		34 ± 1				
Pearl millet thick porridge + *Cratotheca spp* leaves Rf value 0.82		411 ± 1		201 ± 2			
Pearl millet thick porridge + *C. maxima* leaves Rf value 0.94		10 ± 3			470 ± 3		
Pearl millet thick porridge + *B. pilosa* leaves Rf value 0.50						3 ± 1	112 ± 2
Rf value 0.68	233 ± 3		107 ± 5				
Finger millet thick porridge + *C. anguria* leaves Rf value 0.71	620 ± 3						
Finger millet thick porridge + *A. spinosus* leaves Rf value 0.83		128 ± 1					
Finger millet thick porridge + *C. gynandra* leaves Rf value 0.72	300 ± 3						
Rf value 0.88					139 ± 2		
Finger millet thick porridge + *Cratotheca spp* leaves Rf value 0.89					247 ± 6		
Finger millet thick porridge + *C. maxima* leaves Rf value 0.90					180 ± 3		
Finger millet thick porridge + *B. pilosa* leaves Rf value 0.86		128 ± 2		23 ± 3			
Sorghum thick porridge + traditional chicken stew Rf value 0.59			220 ± 1				35 ± 2
Sorghum thick porridge + *A. spinosus* leaves; R_f value 0.79				386 ± 2			
Rice thick porridge + *V. unguiculata* leaves; Rf value 0.78				321 ± 2			
Unpolished *Z. mays* thick porridge + *C. maxima* leaves; Rf value 0.77				244 ± 3			
Unpolished *Z. mays* thick porridge + *A. spinosus* leaves; Rf value 0.82		612 ± 5					
Polished *Z. mays* thick porridge *C. gynandra* leaves Rf value 0.67			108 ± 2				

the number of hydroxyl groups that can donate hydrogen to reduce free radicals. Thus results of the present results are important as previous studies have reported that polyphenols from traditional foods may not be beneficial due to complexation to mineral elements (Gupta et al., 2006).

For the ethanolic extract, finger millet thick porridge combined with boiled *C. anguria* leaves as relish (R_f value 0.71) consist of high levels of salicylic acid portraying high antioxidant activity, 620 ± 3 mg/g of sample followed by pearl millet thick porridge and *C. gynandra* leaves at R_f value of 0.70, 507 ± 3 mg/g. Pearl millet thick porridge and *C. anguria* leaves (R_f value 0.70), pearl millet thick porridge and *B. pilosa* leaves (R_f value 0.68), finger millet thick porridge and *C. gynandra* leaves (R_f value 0.72) also consisted of salicylic acid as the phenolic acid exhibiting high antioxidant activity. This is in contrast with ethyl acetate extract. Only one dish, rice thick porridge and *V. unguiculata* boiled leaves (R_f value 0.69) consisted of salicylic acid. The trend was similar for the other phenolics. Thus ethanol is a better solvent for extracting antioxidant phytochemicals than ethyl acetate. This is because ethanol tends to interact more with non-complexed hydroxyl groups through hydrogen bonding. Gallic acid was also found in large quantities in pearl millet thick porridge combined with *Cratotheca* spp. leaves

Table 6. Concentration of antioxidant phytochemicals in ethyl acetate extract.

Dish name	Concentration of antioxidant phytochemicals (mg/g of sample ± SD)						
	Salicylic acid	Gallic acid	Caffeic acid	Chlorogenic acid	Vanillic acid	Quercetin	Kempferol
Pearl millet thick porridge + *A. spinosus* leaves; Rf value 0.14						112 ± 3	
Pearl millet thick porridge + *C. maxima* leaves; Rf value 0.53						20 ± 3	184 ± 2
Pearl millet thick porridge + *B. pilosa* leaves Rf value 0.76				377 ± 5			
Finger millet thick porridge + *C. anguria* leaves; Rf value 0.94					198 ± 5		
Finger millet thick porridge + *A. spinosus* leaves; Rf value 0.85		132 ± 5					
Finger millet thick porridge + *C. gynandra* leaves; Rf value 0.76				221 ± 3			
Finger millet thick porridge + *C. maxima* leaves; Rf value 0.90					279 ± 2		
Finger millet thick porridge + *B. pilosa* leaves Rf value 0.80		139 ± 1					
Rice thick porridge + *V. unguiculata* leaves Rf value 0.69	53 ± 1						
Unpolished *Zea mays* thick porridge + *C. maxima* leaves Rf value 0.83		98 ± 3					
Polished *Zea mays* thick porridge *C. gynandra* leaves; Rf value 0.96					23 ± 3		

and in unpolished *Z. mays* thick porridge with *A. spinosus* leaves at a concentration of 411 ± 1 and 612 ± 5 mg/g respectively. Generally for both ethyl acetate and ethanolic extracts the traditional dishes studied in the present study consist of more phenolic acids than polyphenols. It is important to note that polished maize thick porridge and meat and fried boiler chicken and Irish potato chips exhibited no antioxidant activity. These dishes are considered to be the food of the affluent while traditional dishes as food of the poor people living in rural areas (Mavengahama et al., 2013). High temperatures involved in frying results in formation of free radicals which may diminish natural antioxidants in foods as they are involved in removing them (Castro et al., 2011; Mariutti et al., 2008). Previous studies have shown that addition of vegetables improved the quality of meat based foods (Mariutti et al., 2011)

Presence of significant amounts of antioxidant phytochemicals in traditional dishes means that they are good candidates for fighting chronic diseases. The importance of traditional dishes has remained until recently largely unacknowledged and unrecognized by the common man, policy makers and nutritionist. This is because there is a general lack of studies discussing the importance of traditional dishes to human health (Flyman and Afolayan, 2006). Although many websites discuss the importance of eating traditional foods scientific information to back the claims are still limited. Results of the present study reveal that there is a need to promote consumption of traditional foods and their incorporation in national food baskets. Instead of them being taken as supplements or alternatives in case of unavailability of 'exotic dishes' (Kepe, 2008) they should be considered as an integral part of the main meal. Traditional dishes carry food for the poor or famine food tags hence they are unpopular to majority of people especially the young ones. Some people would not eat traditional dishes when exotic foods are available. The current philosophy about traditional dishes prohibits their cultivation. They are only harvested from the forest. This is exploitative and unsustainable and may results in decline of the species. It could lead to genetic erosion and possible loss of biodiversity (Bharucha and Pretty, 2010). There is a need therefore for more studies in order to encourage cultivation and utilization of traditional foods.

Conclusion

Traditional dishes studied in the present study consist of higher amounts of antioxidant phytochemicals as compared to spiced fresh chips and broiler chicken meat. Levels of antioxidant phytochemicals depend on the thick porridge and relish combination chosen. Wild vegetables offer better results as compared to meat as relish only. Polished maize thick porridge and meat consist of low amounts of antioxidant phytochemicals. No dish consisted of all antioxidant phytochemicals therefore eating a

variety of them will offer better results. Presence of antioxidant phytochemicals shows that eating traditional dishes frequently may result in reduced cases of free radicals induced chronic diseases such as cancers, diabetes type 2, hypertension and obesity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Comparison of *in vitro* antioxidant activity of some selected seaweeds from Algerian West Coast

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***In vitro* antioxidant activity of methanolic (ME) and ethanolic (ET) extracts of six species of seaweeds (*Cystoseira stricta, Cystoseira compressa, Corallina elongata, Porphyra umbilicalis, Enteromorpha compressa* and *Ulva lactuca*) collected from Algerian West Coast was evaluated, using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay and reducing power assay. They were tested for total phenolic content. The highest phenolic content was observed in *C. compressa* [10.24 ± 0.09 mg gallic acid equivalent (GAE) g^{-1} dry weight (DW) in ME extract and 15.70 ± 0.72 mg GAE g^{-1} DW in ET extract]. In general, the ET seaweed extracts were the most effective fractions. In addition, *C. compressa* and *C. stricta* extracts showed the highest antioxidant activity in all assays and better than vitamin E. However, these extracts present lower DPPH radical scavenging activity and reducing power than butylhydroxytoluene (BHT), except at concentration of 1 mg mL^{-1}, in which it was similar. Therefore, *C. compressa* and *C. stricta* had a potential to be used as a natural antioxidant agent.**

Key words: Algerian, antioxidant activity, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging, reducing power, seaweed.

INTRODUCTION

The marine flora and fauna constitute precious biological resources. The importance of these resources is due to the presence of marine organism having a wide range of metabolites which can be rare or even absent in animals, vegetables, mushrooms or microorganisms. For several years, a particular look has been concerned with the research for new substances of biotechnological interests. In the international pharmaceutical market and cosmetic,

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30% of active substance was developed from natural substances among which 10% was isolated from marine organisms (Ruiz, 2005).

Among these, seaweeds are an important source of bioactive compounds such as carotenoids, dietary fiber, protein, essential fatty acids, vitamins and minerals and many active substances like lipids, proteins, poly-saccharides and polyphenols (Meenakshi et al., 2011). Having many activities, anti-protozoan, antibacterial, anti-inflammatory, anticoagulative, antifungal, anti-free radical, antioxidant, etc (Hellio et al., 2004; Cox et al., 2010; Taboada et al., 2012) seem promising for the pharmaceutical, cosmetics and food industries.

An outbreak of interest is on the biological effects of natural antioxidants, included in the fight against oxidative stress that causes ageing, the release and progress of several diseases such as cancer, cardiovascular accidents, inflammatory diseases and neurodegenerative diseases (Favier, 2003). To prevent the toxic effects of oxygen, several species of seaweeds containing a wide specter of phytochemical substances, which are sources of natural antioxidants agents, such as phenolic acids, flavonoids and tannins, have the capacity to eliminate the reactive species of oxygen (RSO). They can thus minimize the oxidative damage caused by these RSOs in living cells and prevent oxidative degradation of food while supplying simultaneously remarkable nutritional advantages (Cornish and Garbary, 2010). These seaweeds also offer to industrial domain an interesting alternative to synthetic antioxidant substances as butylhydroxyanisol (BHA), butylhydroxytoluene (BHT) and propyl gallate (PG) which are reported carcinogenic (Safer and Al-Nughamish, 1999).

Algeria with its long coastal constitutes a rich but a useless source of marine algae. Indeed, there are few studies estimating the antioxidant potential of seaweeds in Algeria and even less industrial exploitation. These seaweeds constitute a pathway of economic development. In this regard, the aim of this study was to investigate the antioxidant activity of seaweed extracts of various species from Algerian West Coast: *Cystoseira stricta* (Montagne) Sauvageau, *Cystoseira compressa* (Esper) Gerloff & Nizamuddin, *Corallina elongata* Ellis & Solander, *Porphyra umbilicalis* Kützing, *Enteromorpha compressa* (Linnaeus) Nees, and *Ulva lactuca* Linnaeus, with the prospect of a valuation.

MATERIALS AND METHODS

Sample collection

Six species of fresh seaweeds were collected from Algerian West Coast: Division Pheaophyte: *C. stricta* and *C. compressa*; Division Rhodophyta: *C. elongata* and *P. umbilicalis*; and Division Chlorophyta: *E. compressa* and *U. lactuca*. These seaweeds were cleaned from epiphytes, salt and dried at room temperature (23 ± 2°C) for 72 h. The dried samples were powdered and stored at -20°C until used for further experiments.

Preparation of seaweeds extracts

Samples were extracted with two different solvents methanol (ME) and ethanol (ET), according to the method of Kelman et al. (2012) modified. Five grams of each sample was extracted with 100 ml of solvent for 24 h with mixing, at room temperature (25°C) and under dark condition. Resultant extracts were filtered and solvent removed under reduced pressure to yield dry material. Extracts weights were recorded and the yield expressed as percentage.

Determination of total phenolic content

Total phenolic content of each seaweed extract was quantified according to the method of Folin-Ciocalteu (Singleton and Rossi, 1965). A volume of 400 µl of each seaweed extract (at a concentration of 2 mg ml^{-1}) was added to 2 ml of the reactive of Folin-Ciocalteu (diluted 10 times). The mixture was allowed to incubate for 4 min at room temperature. Then, 1.6 ml of Na_2CO_3 (7.5 %) was added. Tubes were shaken and incubated for 2 h in darkness, at room temperature. Samples' absorbencies were read at 765 nm. Gallic acid was used as the standard for a calibration curve. The total phenolic contents of seaweed extracts were expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW).

DPPH radical scavenging assay

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity of samples was performed according to the method of Yen and Chen (1995). Aliquot (2 ml) of test sample (at a concentration of 0.1, 1 and 2 mg ml^{-1}) was added to 2 ml of 0.16×10^{-3} mol L^{-1} DPPH methanolic solution. The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark, and its absorbance was read at 517 nm. The ability to scavenge DPPH radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [1 - (A_{sample} - A_{sample\ blank}) / A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the control (DPPH solution without sample), A_{sample} is the absorbance of the test sample (DPPH solution plus test sample), and $A_{sample\ blank}$ is the absorbance of the sample (sample without DPPH solution). Then, the IC50 (concentration of 50% inhibition of the radical DPPH) was calculated. Vitamin E and BHT were used as positive control.

Reducing power

The reducing power was measured as described by Oyaizu (1986). 1 ml of each extract (at a concentration of 2 mg ml^{-1}) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium hexacyanoferrate solution. After 30 min of incubation at 50°C, 2.5 ml of 10% trichloroacetic acid was added. The mixture was centrifuged at 3000 rpm, for 10 min. Finally, 2.5 ml of the upper layer were mixed with 2.5 ml of distilled water and 0.5 ml, 0, and 1% $FeCl_3$. The absorbance was measured at 700 nm. Vitamin E and BHT were used as positive controls.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD) and are statistically compared by one-way variance analysis (ANOVA) and Tukey HSD test (for homogeneous variance) and by non-parametric variance analysis of Kruskal-Wallis and Mann-Withney U-test (for non homogeneous variance), after verification of variance homogeneity by Hartley test.

Table 1. Yield (%) and total phenolic content (mg GAE g^{-1} DW) of methanolic and ethanolic extracts.

Seaweed	Yield (%)		Total phenolic content (mg GAE g^{-1} DW)	
	ME extract	ET extract	ME extract	ET extract
Cystoseira stricta	8.41 ± 0.26^{a}	3.81 ± 0.13^{a}	8.24 ± 0.89^{a}	4.63 ± 0.21^{a}
Cystoseira compressa	20.26 ± 0.45^{b}	8.89 ± 0.11^{b}	10.24 ± 0.09^{b}	15.70 ± 0.72^{b}
Corallina elongata	2.53 ± 0.90^{c}	1.70 ± 0.55^{de}	4.58 ± 0.14^{c}	3.09 ± 0.16^{c}
Porphyra umbilicalis	5.19 ± 0.23^{d}	2.55 ± 0.09^{c}	3.80 ± 0.05^{d}	4.56 ± 0.02^{a}
Enteromorpha compressa	4.14 ± 0.20^{e}	1.82 ± 0.42^{d}	3.94 ± 0.28^{cd}	3.62 ± 0.16^{c}
Ulva lactuca	9.29 ± 0.16^{f}	1.15 ± 0.24^{e}	2.25 ± 0.05^{e}	3.63 ± 0.06^{c}

Each value is the mean ± standard deviation (n = 3). Different lowercase letters indicate significant difference between means (p < 0.05).

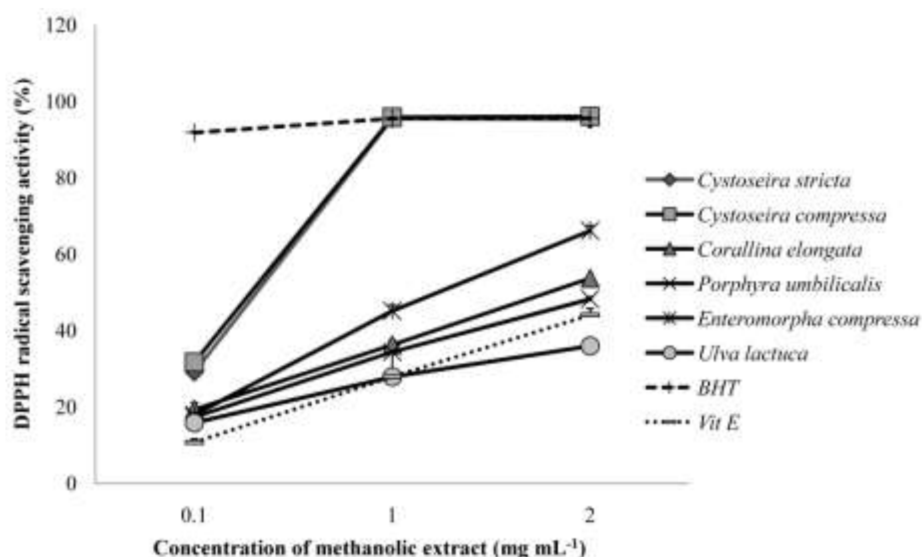

Figure 1. DPPH radical scavenging activity (%) of methanolic seaweeds extracts and positive control (BHT and Vit E), at various concentrations.

RESULTS

Extracts yield

Six species of seaweeds were extracted with methanol and ethanol. The yields of ME extracts recorded were superior to those of ET extracts ($p < 0.05$), except for *C. elongata* in which no effect of extraction solvent was shown (Table 1). A significantly higher yields were obtained in *C. compressa* (20.26% in ME extract and 8.89% in ET extract).

Total phenolic content

Phenolic contents of seaweeds extracts were evaluated and presented in Table 1. No significant effect of both organic solvents on the capacity of extraction of these compounds was recorded, except only in *E. compressa*. Ethanol can be considered as an excellent solvent of extraction ($p < 0.05$) in *C. compressa, P. umbilicalis* and *U. lactuca*. The brown seaweeds of *Cystoseira* genus showed higher phenolic contents, with the best amount in *C. compressa* (10.24 mg GAE g^{-1} DW in ME extract; 15.70 mg GAE g^{-1} DW in ET extract).

DPPH radical scavenging activity

Percentage of inhibition and IC50 of six seaweeds are presented in Figures 1 and 2 and Table 2. No significant effect of extraction solvents was recorded, except for ethanol, in *C. stricta, C. compressa, E. compressa* and *U. lactuca* at 0.1 mg ml^{-1} and in *P. umbilicalis* at 2 mg ml^{-1} ($p < 0.05$). Moreover, the extracts from *Cystoseira* genus showed the highest scavenging activity ($p < 0.05$) in both solvents (Figures 1 and 2). The ET extract of *C. stricta* recorded an IC50 of 0.1 mg ml^{-1} followed by *C. compressa*

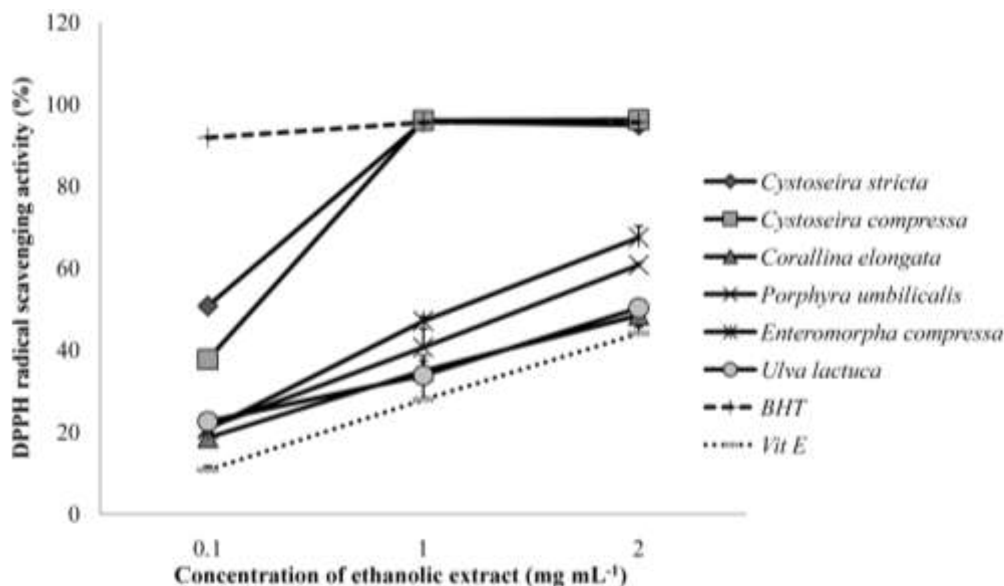

Figure 2. DPPH radical scavenging activity (%) of ethanolic seaweeds extracts and positive control (BHT and Vit E), at various concentrations.

Table 2. IC50 (mg mL^{-1}) of methanolic and ethanolic extracts.

Seaweed	IC50 (mg ml^{-1})	
	ME extract	**ET extract**
Cystoseira stricta	0.35	0.10
Cystoseira compressa	0.29	0.15
Corallina elongata	1.78	2.14
Porphyra umbilicalis	2.08	1.53
Enteromorpha compressa	1.32	1.26
Ulva lactuca	3.45	1.91
BHT	0.05	
Vit E	2.38	

with an IC50 of 0.15 mg ml^{-1} (Table 2). All seaweed extracts showed higher anti-radical activity than vitamin E and lower than BHT ($p < 0.05$), except *C. compressa* and *C. stricta* extracts at concentration of 1 and 2 mg m^{-1} that showed a similar percentage of inhibition of the radical DPPH than BHT.

Reducing power

Figure 3 presented results of reducing power. A significantly higher reducing activity was reported in ET extracts of all seaweeds ($p < 0.05$). Moreover, seaweeds of *Cystoseira* genus showed a maximum absorbance (OD) value in both organics extracts ($p < 0.05$): *C. compressa* (OD = 1.32) in ET extract and *C. stricta* (OD = 0.87) in ME extracts. The positive control BHT showed higher reducing power (OD = 5.18) than all seaweed samples and Vitamin E (OD = 0.079).

DISCUSSION

In the present study, the extraction yield in ME extracts was higher than ET extracts. These results are in agreement with those of Lopez et al. (2011), which showed that the yield of ME extract of brown alga was higher than that obtained with ethanol. Matanjun et al. (2008) noticed that ME extracts of red and green seaweeds had a higher yield, compared with those of diethylether and reported that more polar compounds were found in seaweed extracts and increasing solvent polarity increased the extraction yield (Boonchum et al., 2011).

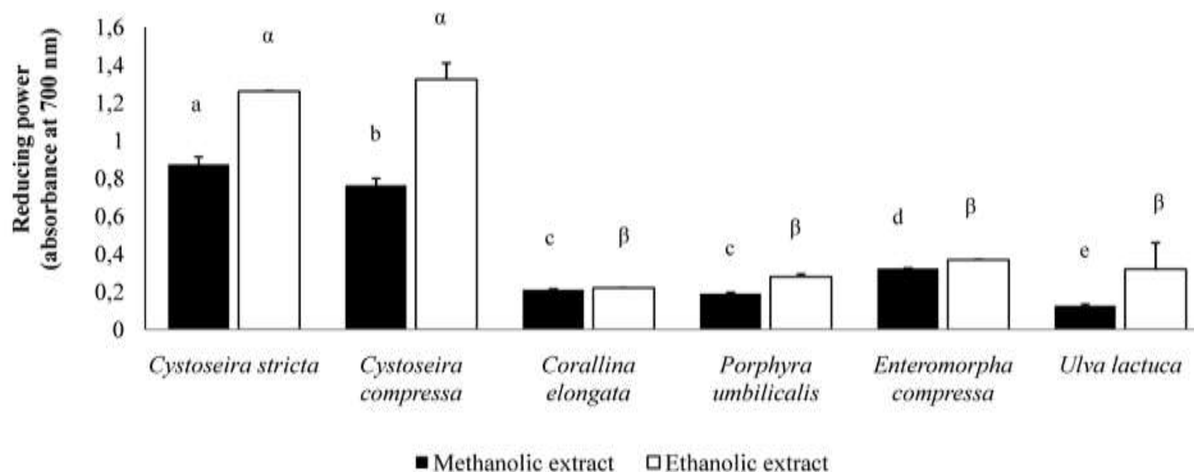

Figure 3. Reducing Power of methanolic and ethanolic seaweeds extracts (concentration of extract used = 2 mg mL^{-1}). Each value is the mean ± standard deviation (n = 3). Different lowercase letters indicate significant difference between means ($p < 0.05$).

Marine seaweed substances, especially polyphenols have antioxidant activity and the major active compounds are phlorotannins and fucoxanthin (Yan et al., 1999). In the polar organic solvents, the phenolic compounds are generally more soluble than in water. The recommended effective solvents are methanol, ethanol and acetone (Waterman and Mole, 1994). By polar solvents, phenolic compounds which were attached in sugars or proteins, saponines, glycosides, organic acids, salts, and in the mucus can be extracted (Cho et al., 2007). The most important contents in P.T were found in the brown seaweeds *Cystoseira* from Algerian West Coast. Matanjun et al. (2008) observed the same result. The brown seaweeds (*Dictyota dichotoma, Sargassum polycystum* and *Padina* species) contained higher phenolic content than the green (*Caulerpa lentillifera* and *Caulerpa racemosa*) and red seaweeds (*Eucheuma cottonii*, *Eucheuma spinosum* and *Halymenia durvillaei*).

Similar phenolic amounts to those observed were reported in *C. elongata* (4.43 ± 4.01 mg GAE g^{-1} DW) (Rico et al., 2012), *Porphyra tenera* (4.70 ± 0.60 mg GAE g^{-1} DW) (Machu et al., 2015), *Enteromorpha intestinalis* (2.65 ± 0.08 mg GAE g^{-1} DW), *U. lactuca* (2.36 ± 0.07 mg GAE g^{-1} DW) (Farvin and Jacobsen, 2013), *C. stricta* (10.14 ± 0.16 mg GAE g^{-1} DW) (Guezzen, 2014); *Cystoseira myrica* (10.08 ± 1.13 mg GAE g^{-1} DW) (Sadati et al., 2011), and *Cystoseira tamariscifolia* (10.91 ± 0.07 mg GAE g^{-1} DW) (Zubia et al., 2009).

Other works reported different phenolic contents. Ganesan et al. (2011) observed higher amount in *E. compressa* (7.76 ± 0.10 mg GAE g^{-1} DW); in *C. compressa,* Güner et al. (2015) reported 0.16 ± 0.08 mg GAE g^{-1} DW, and Mhadhebi et al. (2014), reported 61 ± 0.30 mg GAE g^{-1} DW. This difference is probably due to the very variable properties of every species, which are in connection with the extrinsic factors (irradiation, salinity, nutriments, season, site of sampling and its depth) and intrinsic factors (stage of development and reproduction of seaweed). Our results also indicate that the higher antioxidant activity was observed in extract with higher phenolic contents which are in agreement with Duan et al. (2006) and Boonchum et al. (2011).

Screening of antioxidant activity by DPPH radical scavenging activity was well used by authors because it can be used for various samples and a low amount of active substances can be detected (Sanchez-Moreno, 2002). Our results showed an anti-radical activity which is dependent-dose. In fact, Ismail and Tan (2002) and Cho et al. (2011) reported that the reduction of radical DPPH increased significantly according to the increase of concentrations of seaweeds extracts and positive controls. In general, results indicate that ethanol has a better effect than methanol on the anti-radical activity (Ismail and Tan, 2002). In agreement with Güner et al. (2015) and Mhadhebi et al. (2014), the genre *Cystoseira* revealed a strong anti-radical activity, at various concentrations. Parthiban et al. (2014) also reported that brown algae showed significantly higher phenolic content and antioxidant activities than the red and green seaweeds. *C. stricta* recorded strong IC50 of 0.1 mg ml^{-1} compared to those found in *Cystoseira crinita* (20 ± 0.5 µg ml^{-1}), *Cystoseira sedoides* (50.3 ± 0.1 µg ml^{-1}) and *C. compressa* (61 ± 0.3 µg ml^{-1}) (Mhadhebi et al., 2014). In addition, the capacity of all seaweed extracts to inhibit the DPPH radical is lower than that of positive control BHT (Ismail and Tan, 2002; Shanab et al., 2011), except, the anti-radical activity of *Cystoseira,* that remains comparable to that of BHT at 1 and 2 mg ml^{-1}. However, vitamin E showed an anti-radical activity lower than that of BHT and the various studied seaweeds. This is also

reported by Narasimhan et al. (2013). This suggests the use of this seaweeds species as a better source of polyphenols and it is more advantageous than vitamin E.

The brown algae of *Cystoseira* genus present a better reducing power, with an influence of the solvent of extraction. Luo et al. (2010) also reported the highest amount of reducing power in brown seaweeds. The ET extracts have a reducing power superior to that of methanolic extracts. Cho et al. (2010) also related that the most effective reducing power is obtained from ET extracts of the green algae *E. compressa* and *Capsosiphon fulvescens*. Ethanol is a solvent that is not toxic and more interesting for industrialists. Our results revealed also that green alga *E. compressa* had a capacity to reduce the iron higher than that of red seaweeds (*C. elongata* and *P. umbilicalis*). These results are in agreement with those of Zhang et al. (2007) and Narasimhan et al. (2013) which related that the reducing power of green seaweeds is superior to that of the red seaweed. In comparison with the positive control (BHT and vitamin E), our results indicate that the reducing capacity of our extracts is lower than that of the BHT and superior to that of the vitamin E. It was also reported by Shanab et al. (2011), who showed that the capacity to reduce the iron by BHT is higher than that of the green alga *E. compressa*. Ganesan et al. (2008) and Cho et al. (2010) observed that reducing capacity of the red seaweed *Euchema kappaphycus* and green seaweed *E. compressa* is higher or comparable to that of the vitamin E.

The BHT revealed an antioxidant capacity superior to those of the seaweeds. However, this synthetic antioxidant has proved toxic and carcinogenic (Safer and Al-Nughamish, 1999). So, it must be replaced by safe and little expensive natural antioxidants.

In conclusion, the brown algae *Cystoseira* collected from Algerian West Coast revealed the best anti-radical and reducing capacities, with higher phenolic content among the six seaweeds, in both solvents of extraction. This suggests that methanol and ethanol can be used for extraction of active compounds (in particular the total polyphenols); and preferentially ethanol, which would present less risk in industrial products. Also, it would be interesting to study *in vivo* antioxidant activity of this antioxidant-rich extracts which may be used as a dietary supplementary or as a natural antioxidant in food industries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Interactive effect of colloidal solution of zinc oxide nanoparticles biosynthesized using *Ocimum gratissimum* and *Vernonia amygdalina* leaf extracts on the growth of *Amaranthus cruentus* seeds

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Nanofertilizers may be a possible replacement for organic fertilizers because they can get to targeted sites in a plant and either enhance or thwart its growth depending on the toxicity level of the used nanoparticles. Zinc oxide nanoparticles are said to be a good source of nutrients for crop production and using these nanoparticles as bio fertilizers, could help to reduce the chemical residue in the grown vegetables which may sometimes be consumed raw or at an immature stage. Zinc oxide nanoparticles used in this work were biosynthesized using *Ocimum gratissimum* and *Vernonia amygdalina* leaf extracts. They were characterized using optical spectroscopy and electron microscopy and found to be of sizes ranging from 11 to 99 nm with some agglomeration. Colloidal solutions of these nanoparticles of concentrations 10, 100 and 500 mgl^{-1} were used as nanofertilizer for growing black-seeded and pale-seeded *Amaranthus cruentus*. Percent emergence was best at 69% for the pale seeded variety to which 100 mgl^{-1} Og-ZnO (pH 8) nanoparticles was applied and worst at 29% for the black seeded variety treated with 10 mgl^{-1} Og-ZnO (pH 12) nanoparticles. Best emergence index value of 2.28 was observed for the black seeded variety on the application of pH 8, 100 mgl^{-1} Og-ZnO nanoparticles. The pale seeded variety to which pH 12, 10 mgl^{-1} of Va-ZnO nanoparticles was applied gave the best emergence rate index results. The study showed that pH and concentration of nanoparticles can affect the seedling characteristics of *A. cruentus* seeds.

Key words: Nanofertilizer, percent emergence, emergence index, emergence rate index, seedling characteristics, electron microscopy, optical spectroscopy.

INTRODUCTION

Nanoparticles have sizes in the nanometer range and exhibit novel properties which are dependent on their morphology (Vidya et al., 2013). They can be synthesized using chemical, physical and biological methods but of these three methods, biosynthesis is preferred because of its environmental friendliness (Singh et al., 2011). The use of nanoparticles spans through a number of disciplines ranging from household items to the field of

electronics as sensors. While in medicine they aid drug delivery to specific targets, most recently in agriculture they have been said to play active roles in plant growth (Sabir et al., 2014). Some nanoparticles are reported to have excellent antibacterial and antimicrobial properties and others are said to exhibit considerable phototoxicity on growing plants (Lin and Xing, 2007).

Recent researches have shown that nanoparticles can be used as nanofertilizer for growing plants but though some researchers say nanoparticles thwarts the growth of some plants, others reported growth enhancement of some other plants (Aslani et al., 2014; Sabir et al., 2014). The effect of nanoparticles on plants varies with the kind of nanoparticles used, the plant species under study, the form of the nanoparticles in use and the concentration of its colloidal solution (Ma et al., 2010).

Zinc oxide nanoparticles biosynthesized using *Ocimum gratissimum* (Scent leaf) and *Vernonia amygdalina* (bitter leaf) leaf extracts were used as nanofertilizer for this study. *O. gratissimum* and *V. amygdalina* are both antioxidants with valuable food and medicinal values which contain bio molecules with reductive abilities. While *O. gratissimum* can be used for treating headaches, inflammation, pneumonia, haemorrhoids , common colds and infant convulsions (Akinyemi et al., 2005; Prabhu et al., 2009), *V. amygdalina* serves as a laxative and is used by herbalists for treating malaria probably due its Quinone content. It is also said to be effective for assisting diabetic control of sugar level (Akinjogunla et al., 2011; Katewa et al., 2004; Udochukwu et al., 2015; Ugwoke et al., 2010).

Zinc oxide nanoparticles have some antibacterial properties and a wide range of applications. They can be used to fabricate some functional device structures such as relative humidity sensors due to their resistance change upon exposure to different relative humidity conditions (Erol et al., 2010; Vaseem et al., 2010). Their ability to produce bright UV and visible fluorescence has been used for optical applications such as light emitting devices (LEDs) as well as in the field of bio-medical imaging (Vaseem et al., 2010). Organic fertilizers for growing plants are in high demand and even when available are not enough to meet the growing demand for it, nor do they completely get absorbed by the plants or get to designated sites in the plants due to hydrolysis, leaching etc (Nair et al., 2010).

In contrast, a small quantity of nano fertilizer when applied to plants is said to actually get to the targeted sites in the plants (Sabir et al., 2014) and nano-encapsulated slow release of fertilizers conserve fertilizers and reduce environmental pollution (Nair et al., 2010). Nanoparticles are beneficial to agriculture and can serve as an alternative to organic fertilizer. This, according to Siddiqui et al. (2015), is because nano-particles with high surface areas are highly reactive and can get to targeted sites in a plant in order to enhance its metabolism. Furthermore, surface area and surface properties of nanoparticles (Ma et al., 2010) rather than their concentrations influence phytotoxicity of seedlings.

Ramesh et al. (2014) compared the effect of bulk and nano Zinc oxide (ZnO) and Titanium dioxide (TiO_2) on seed germination, mitosis, seeds, shoot and root growth of wheat (*Triticum aestivum* Linn) and reported that bulk metal oxides had no significant effect on any of those rather nano ZnO raised Chlorophyll and protein levels of treated seeds. A related research (Stampoulis et al., 2009) which studied the effect of bulk and nano forms of five nanomaterials namely silver, copper, zinc oxide, silicon and multiwalled CNTs (MWCNTs on Field pumpkin (*Cucurbita pepo*), observed that seedling germination percentage was higher for seeds treated with nano ZnO than those treated with bulk ZnO powder and that percent emergence of the seeds was dependent on the sizes of the nanoparticles used for treatment of seeds.

Nanoscale metals such as zinc or aluminum or metal oxides like ZnO or TiO_2 have been applied to selected plants and their toxicology effects on the seeds, roots or growth have been studied. While TiO_2 is said to shield plant Chlorophyll from destruction due to long exposure to light, it has also been mixed with SiO_2 and applied to soybean to boost its antioxidant effect and intake of water and other essential nutrients (Lu et al., 2001).

While studying the toxicity effects of five nanoparticles (MWCNT, Al_2O_3, ZnO, Al, and Zn) with concentrations ranging from 10 mgl^{-1} to as high as 2000 mgl^{-1} on six plants (rape, radish, rye grass, lettuce, corn and cucumber), the researchers (Lin and Xing, 2007) reported that none of the applied nanoparticles showed inhibition to growth except for nano zinc, which affected rye grass and nano zinc oxide which affected corn.

While these researches show the extent of phytotoxicity experienced by the studied plants due to the applied nanoparticles, none of them addressed the interactive effect of nanoparticles of various pH values and their concentration on the seedling characteristics of their chosen species of plants.

This work will not discuss the biological process behind the assimilation of the zinc oxide nanoparticles into the plant cells but will dwell on the physical characteristics of the nanoparticles and how the concentration of the ZnO nanofertilizer and various seed treatments make observable changes in the seedling characteristics of the *Amaranthus cruentus* seeds. It will also show how these changes depend on the pH of the synthesized zinc oxide nanoparticles and the concentration of its colloidal solution.

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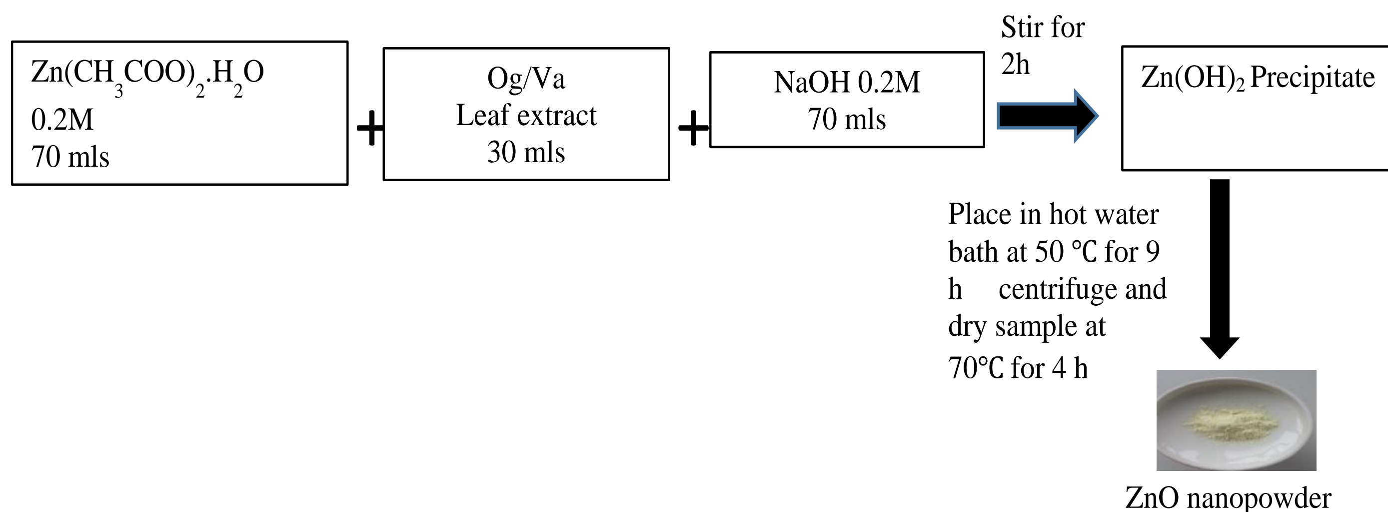


Figure 1. Schematic illustration of pathway of the ZnO nanoparticles synthesis.

MATERIALS AND METHODS

The ZnO nanoparticles were biosynthesized using leaf extracts of *O. gratissimum* and *V. amygdalina* and then characterized using UV-vis, scanning electron microscope (SEM), Transmission electron microscope (TEM), XRD, Fourier transform infrared spectroscopy (FTIR) and PL studies. Colloidal solutions of these nanoparticles of different concentrations were prepared and used as fertilizer for growing black-seeded and pale - seeded *A. cruentus*. The interactive effect of pH of the nanoparticles of different concentrations on the seedling characteristics of these seeds namely percent emergence (%E). Emergence Index (EI) and Emergence rate index (ERI) of these two varieties of *A. cruentus* were investigated.

A. cruentus which belongs to a family of plants called *Amaranthaceae* is used in place of spinach in some countries. It is said to have grains which are rich in protein and leaves which are rich in vitamins. It is cheap, common, and grows generally well in the tropics.

Nanoparticles synthesis and colloidal solution preparation

Two samples of zinc oxide nanoparticles were biosynthesized using 15% leaf extracts of *O. gratissimum* (Og) and *V. amygdalina* (Va) with zinc acetate dihydrate and NaOH as precursor materials (Sidra et al., 2014) and the resulting precipitate was kept in a hot water bath at 50°C for 9 h after which it was centrifuged and dried in an oven for 7 h (Figure 1).

Three samples each of Og-ZnO and Va ZnO nanoparticles of pH values P_1= pH 8, P_2= pH 10 and P_3=pH 12 were synthesized and characterized using optical spectroscopy and electron microscopy. Colloidal solutions of the zinc oxide nanoparticles of concentrations N_1=10, N_2=100, and N_3=500 mgl^{-1} were prepared for each sample.

Seeds and their planting

Two varieties of *A. cruentus,* namely, the black-seeded and the pale-seeded varieties were used for the study. These seeds were given some extra treatments before planting: (i) soaked in nanoparticles solution for 10 min (ii) soaked in water for 10 min and (iii) not soaked. The soil used for the planting was analyzed for its constituent elements before planting and after harvesting the plants.

The soil was of pH 6.0 and consisted of 79.5% of sand, 10.5% of clay and 10.0% of silt. Organic carbon was 1.50%, and its zinc content was 0.24 mg kg^{-1}. The pots used for the seed planting each contained soil of volume $8×10^{-3}$ m^3 and the seeds were planted in pots at a depth of 0.7 cm. Each treatment had three replications and the blank control pots had soil with no nanoparticles treatment. A screen house was used for shielding the pots, used for the seed planting from pests to ensure that only the nanoparticles played a role in seed germination. The International Seed Testing Association (ISTA) method in which 100 seeds are planted in each pot was used and after emergence the plants were thinned to eight with four of those actually tagged for all the measurements that were done. The study of the seedling germination was done over a period of one week and the parameters of interest were the percent emergence % E the emergence index EI, and the emergence rate index ERI of the planted seeds. Four factors were introduced in the experiment namely (i) pH of synthesized nanoparticles (ii) seed varieties (iii) concentration of colloidal solutions of the zinc oxide nanoparticles (iv) seed soaking.

Preparation of zinc oxide nanofertilizer

The different concentrations of the colloidal solutions of the zinc oxide nanoparticles were prepared and labelled accordingly with pH 8, 10 and 12 referred to as P1, P2 and P3, respectively. The concentrations of solutions prepared were 10, 100 and 500 mgl^{-1} referred to in this report as N1, N2 and N3, respectively. The zinc oxide nanoparticles solution is used as nanofertilizer in this experiment. The soil in the pots in which the seeds were to be planted was treated with the nanofertilizer before planting and was repeated a week after seed emergence. The blank control experiments pots were designated as performance of the blank control (P0N0) and had soil with no nanoparticles treatments.

Statistical analysis

Data of the interactive effect of nanoparticles pH and concentration on the seedling characteristics was subjected to the analysis of

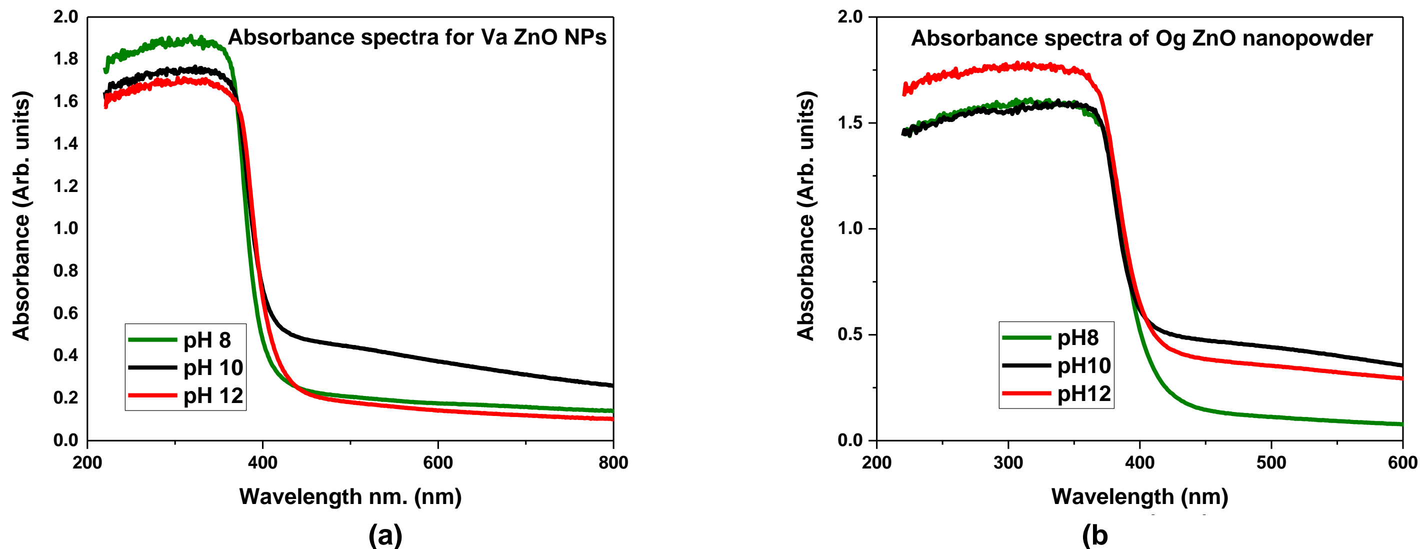


Figure 2. (a) Absorbance spectra of the Va and (b) Og zinc oxide nanopowder.

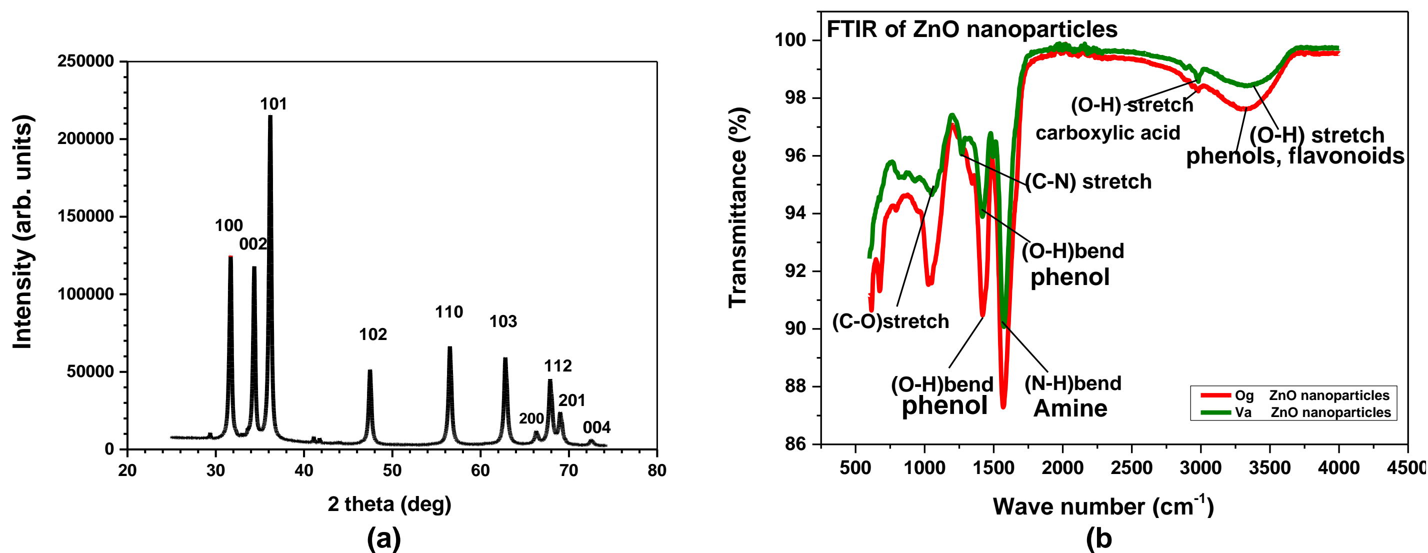


Figure 3. (a) XRD pattern and (b) FTIR scan results of the zinc oxide nanopowder.

variance (ANOVA) and tests were done at 5% probability using the Genstat release 10.3 DE. The effects of soaking the seeds before planting were however ignored because it was not significant.

RESULTS AND DISCUSSION

Characterisation of zinc oxide nanoparticles

The absorbance spectrum for zinc oxide nanopowder (Figure 2) shows that the Va sample interacts better with light than the Og sample, with a higher peak absorbance at wavelength 355 nm as against 360 nm for Og sample. The absorbance increased with pH for Og zinc oxide nanoparticles but the reverse is true for Va zinc oxide nanoparticles. While the XRD pattern of the ZnO nanopowder (Figure 3a) shows that it has the hexagonal wurtzite structure, the FTIR spectra of zinc oxide nanoparticles (Figure 3b) revealed the functional groups on the nanoparticles surface, which are present in flavonoids, phenols, amides and polysaccharides. These biomolecules may have been involved in the reduction of the zinc ions to nanoparticles.

The near-ultraviolet (NUV) Photoluminescence spectra of the zinc oxide nanoparticles (Figure 4) show two

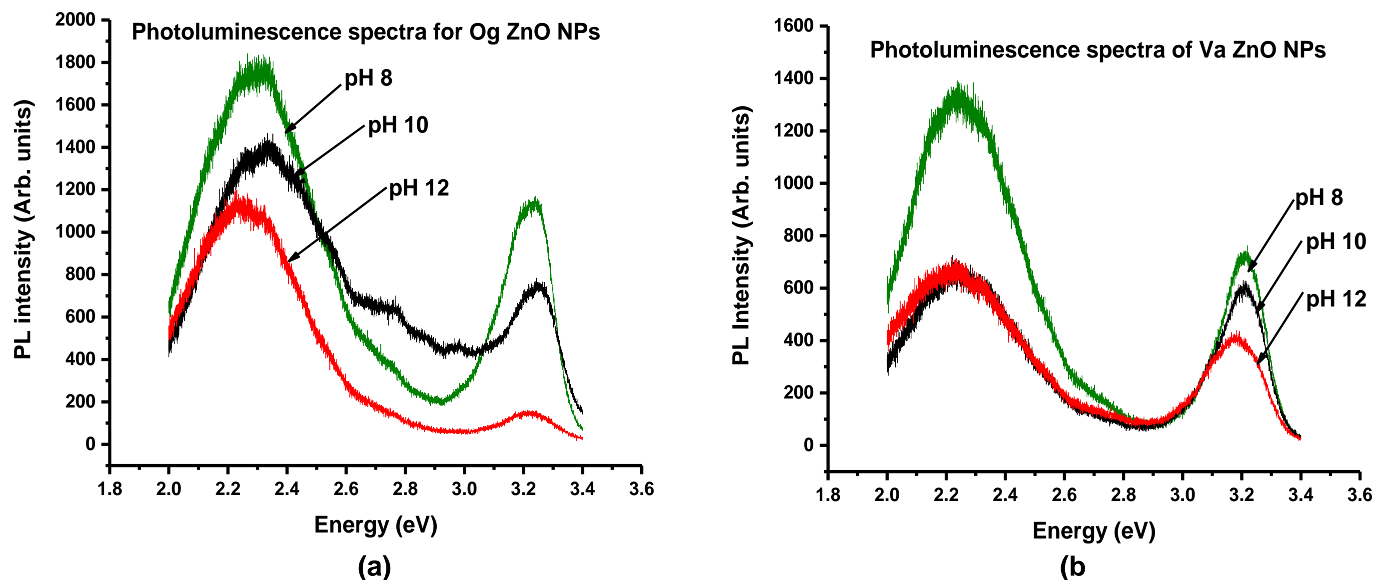


Figure 4. (a) Photoluminescence spectrum of the ZnO nanopowder of different pH values for (a) Og and (b) Va.

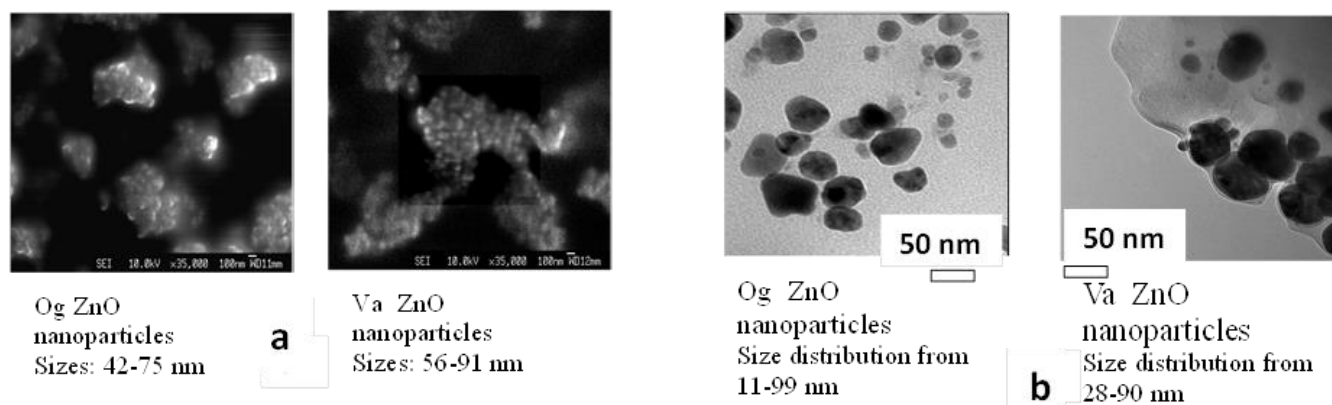


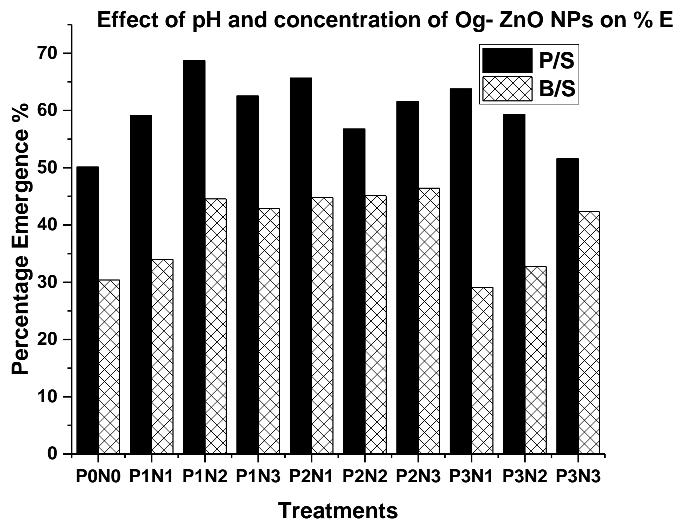
Figure 5. (a) SEM micrographs and (b) TEM images of the zinc oxide nanopowder.

emission peaks. Lower wavelength peaks attributed to the near bandgap excitonic emission and higher wavelength peaks due to singly ionized oxygen vacancies (Sangeetha et al., 2011). These have values ranging from 381 to 384 nm for pH 8 to 10 Og - zinc oxide nanoparticles and 532 to 551 nm while for the Va - zinc oxide nanoparticles, the values were in the range 386 to 390 nm and 553 to 556 nm, respectively. The PL intensities were found to decrease for higher pH samples. The SEM images (Figure 5a) show the particles to be spherical with aggregation and with low pH particles larger in size than higher pH nanoparticles.

The Og pH 8 zinc oxide nanoparticles had sizes ranging from 42 to 75 nm, giving an average size of 63 nm while the pH 10 nanoparticles had sizes ranging from 36 to 55 nm and an average size of 43 nm. The Va zinc oxide nanoparticles of pH 8 had sizes ranging from 56 to 91 nm and an average size of 71 nm while the pH 10 nanoparticles had sizes in the range of 50 to 78 nm and an average size of 54 nm. The TEM images (Figure 5b) show the nanoparticles to be spherical in shape with some aggregation, with sizes ranging from 11 to 99 nm for Og and Va samples.

Seedling characteristics

The number of days to emergence of the planted seeds was two and it took a little above a week for all the viable seeds to germinate. The interactive effects of pH and concentration of the Og and Va zinc oxide nanoparticles, resulting from the analysis of variance (ANOVA) of the



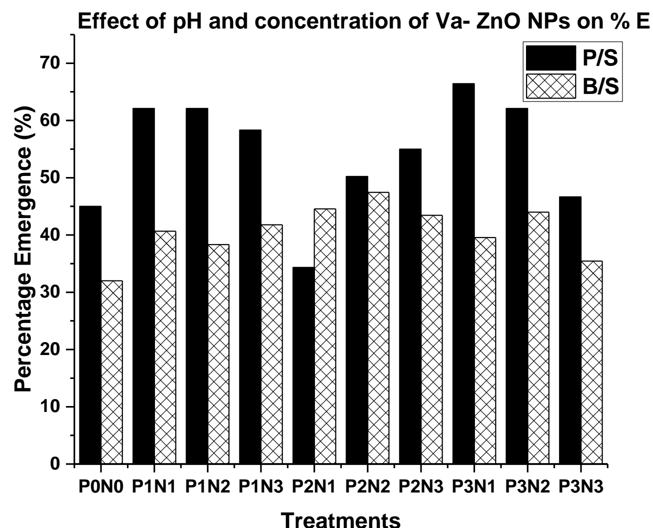
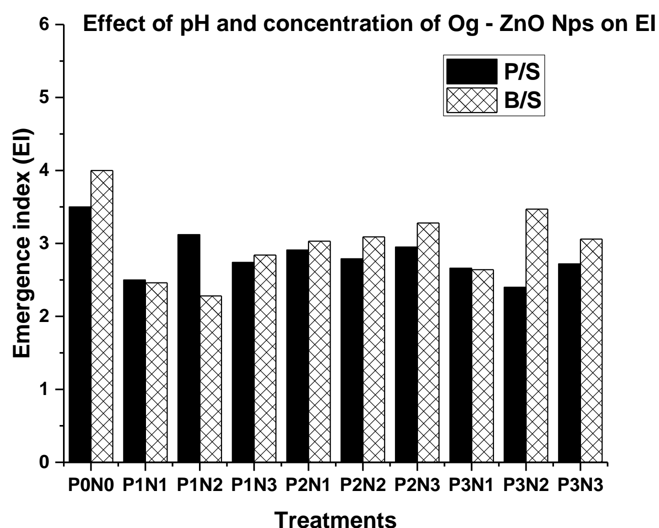


Figure 6. Interactive effect of pH and concentration of colloidal solutions of Og and Va zinc oxide nanoparticles on the percentage emergence of the *Amaranthus cruentus* seed.



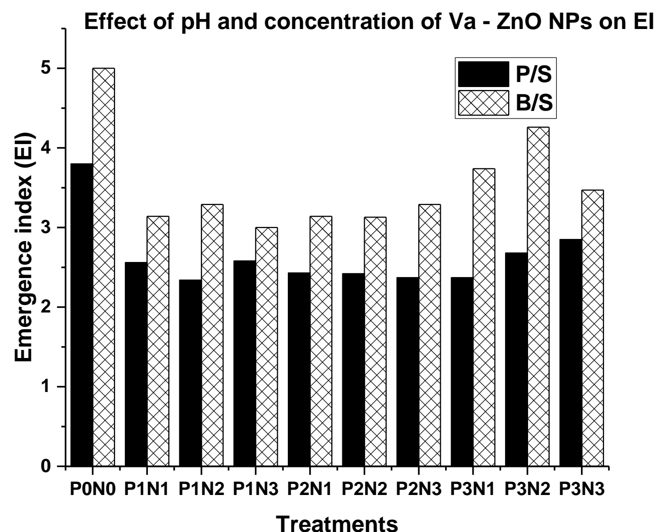


Figure 7. Interactive effect of pH and concentration of colloidal solutions of Og and Va zinc oxide nanoparticles on the emergence index of the Amaranthus cruentus seed.

collected data was done. The blank control P0N0 was compared with those of the treated seeds and the result of the seedling characteristics are as shown in the plotted bar charts (Figures 6 to 8).

Percent emergence (% E)

Percent emergence values give an indication of the number of seeds out of the 100 planted which germinated. High values are considered to be good. For black seeded variety and for all three pH levels, increasing Og-ZnO nanoparticles concentration increased the percent emergence.

For the pale seeded variety however, increasing the pH 8 nanoparticles concentration increased the percent emergence but increasing the pH 10 and 12 nanoparticles concentration, decreased the percent emergence

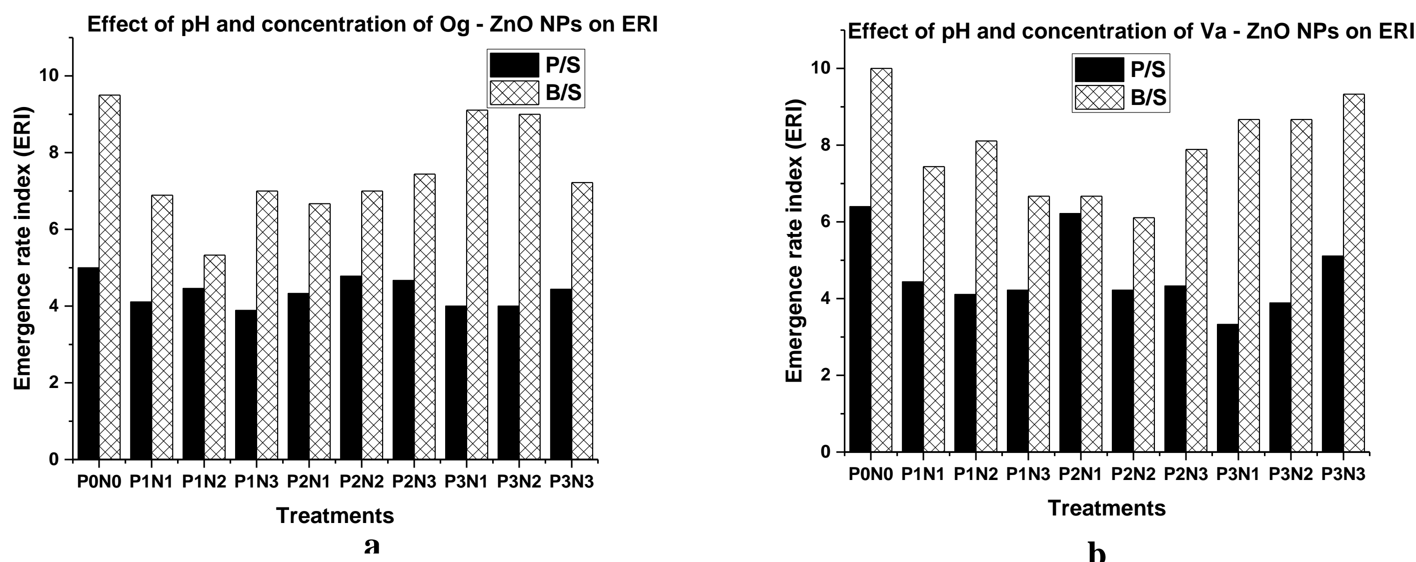


Figure 8. Interactive effect of pH and concentration of colloidal solutions of Og and Va zinc oxide nanoparticles on the emergence rate index of the Amaranthus cruentus seed.

(Figure 6a). Increasing the pH 8 Va-ZnO nanoparticles concentration raised the percent emergence of the black seeded variety but lowered that of the pale seeded variety.

Similarly high concentrations of the pH 10 nanoparticles reduced the black seeded variety percent emergence but increased that of the pale seeded variety. High concentrations of the pH 12 nanooparticles reduced the percent emergence of both the pale and black seeded variety (Figure 6b).

Emergence Index (EI)

The emergence index shows the uniformity in growth of the planted seeds. Low EI values are considered to be better than higher ones. The results for the emergence index (EI) as shown in Figure 7 seem to be equally distributed between the two seed varieties with Og zinc oxide nanofertilizer pH 8, 100 mgl^{-1} giving the best value of 2.28 for the black seeded variety (Figure 7a), and the worst value of 4.26 was recorded for same seed variety treated with pH 12, 100 mgl^{-1}Va zinc oxide nanofertilizer (Figure 7b).

Increased Og ZnO nanoparticles concentration of all three pH levels increased EI values for both the pale and black seeded varieties. Raising the concentration of pH 8 Va ZnO nanoparticles increased the EI values of the pale seeded variety but reduced that of the black seeded variety. High concentrations of pH 10 Va ZnO nanoparticles gave low EI values for pale seeded variety but high EI values for black seeded variety. Furthermore, high concentrations of pH 12 Va ZnO nanoparticles gave better EI values for black seeded variety but poor EI values for pale seeded variety.

Emergence Rate Index (ERI)

Emergence rate index values show on a scale of zero to ten, how long it actually took all the planted seeds to emerge. Low ERI values are preferred to higher ones. Results of the emergence rate index (Figure 8) showed that, the pale seeded variety did better than the black seeded variety when treated with either Og or Va zinc oxide nano fertilizer. However the best result of 3.35 was observed for pH 12, 10 mgl^{-1} Va zinc oxide nanofertilizer (Figure 8b) and the worst result was for the black-seeded variety with ERI 9.36 due to pH 12, 500 mgl^{-1} Va zinc oxide nano fertilizer (Figure 8b).

Increasing the concentration of pH 8 Og ZnO nanofertilizer increased ERI values for the black seeded variety but lowered the ERI values for the pale seeded variety. For both the pale seeded and black seeded variety, increasing the concentration of pH 10 nanoparticles raised their ERI values however, high concentrations of the pH 12 nanoparticles lowered the ERI values of the black seeded variety but increased that of the pale seeded variety, implying that better growth uniformity could be achieved using lower concentrations of nanoparticles of all pH levels.

Conclusion

Zinc oxide nanoparticles of three pH levels were biosynthesized using *O. gratisimum* and *V. amygdalina* x

Table 1. Summary of the treatments that produce good results for species.

Parameter	Og ZnO nano fertilizer				Va ZnO nano fertilizer			
	BT	Seed variety/Results	WT	Seed variety/Results	BT	Seed variety/Results	WT	Seed variety/Results
% E	P1N2	PS (69 %)	P3N1	BS (29 %)	P3N1	PS (67 %)	P2N1	PS (34 %)
E I	P1N2	BS (2.28)	P3N2	BS (3.47)	P1N2	PS (2.34)	P3N2	BS (4.26)
ERI	P1N3	PS (3.89)	P3N1	BS (9.11)	P3N1	PS (3.33)	P3N3	BS (9.33)

PS→ Pale- seeded Amaranth. P1 = pH 8, P2 = pH 10, P3 = pH 12. N1 = 10 mgl^{-1}; N2 = 100 mgl^{-1}; N3 = 500 mgl^{-1}; BS→ Black seeded Amaranth: BT →Best treatments; WT → Worst treatments.

biosynthesized using *O. gratisimum* and *V. amygdalina* and characterized using optical spectroscopy and electron microscopy. They were found to be spherical in shape with sizes which were found to be pH dependent. Higher pH zinc oxide nanoparticles were smaller in size than the lower pH zinc oxide nanoparticles for both the Og and Va zinc oxide nanoparticles. The Va zinc oxide nanoparticles were more sensitive to light giving a higher UV-Vis absorbance peak than the Og zinc oxide nanoparticles, with a lower UV-Vis absorbance peak.

The TEM images showed the nanoparticles to be spherical in shape and of a range of sizes while the SEM micrographs present them as aggregated entities with sizes which were pH dependent. The FTIR scans of the zinc oxide nanoparticles showed functional groups present in the nanoparticles and point to the presence of biomolecules such as flavonoids, phenols and polysaccharides which probably aided the reduction of the zinc ions to zinc nanoparticles.

Colloidal solutions of zinc oxide nanoparticles of three different pH values were used as fertilizer for growing pale and black seeded *A. cruentus*. A significant positive effect of the prepared nanofertilizer on the seedling characteristics was observed as the blank control seeds gave poorer results when compared with the treated seeds. The treatments which resulted in best and worst results are as shown in Table 1. While high concentrations of the Og ZnO nanoparticles of all three pH levels raised the percent emergence of the black seeded variety, high concentrations of the pH 12 Va ZnO nanoparticles and lowered the percent emergence of both seed varieties.

Increased concentrations of Og ZnO nanoparticles of all pH levels gave high EI values and hence poor growth uniformity of the two seed varieties. Lower concentrations of Og ZnO nanoparticles of all pH levels gave the pale seeded variety low ERI values and showed that all seeds of that variety could germinate in four days as against the black seeded variety, which needed as much as ten days for the germination of all its viable seeds. This work showed that pH and concentration of the colloidal solution of the zinc oxide nanoparticles used as nano fertilizer affected the seedling characteristics of the cultivated seeds and corroborated the results of similar researches (Lu et al., 2001; Stampoulis et al., 2009) on other crops, which attest to the effectiveness of nano zinc oxide in seedling growth.

Since none of the zinc oxide nanoparticles treatments choked the planted seeds, the biosynthesized ZnO nanoparticles could be explored for possible use as an active ingredient of a nanofertilizer and could supplement the role of organic fertilizer, whose current supply does not seem to meet demand. Furthermore, this work can chart a new course for the two leaves *O. gratisimum* and *V. amygdalina* used rather than their present use only for food and medicinal values.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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