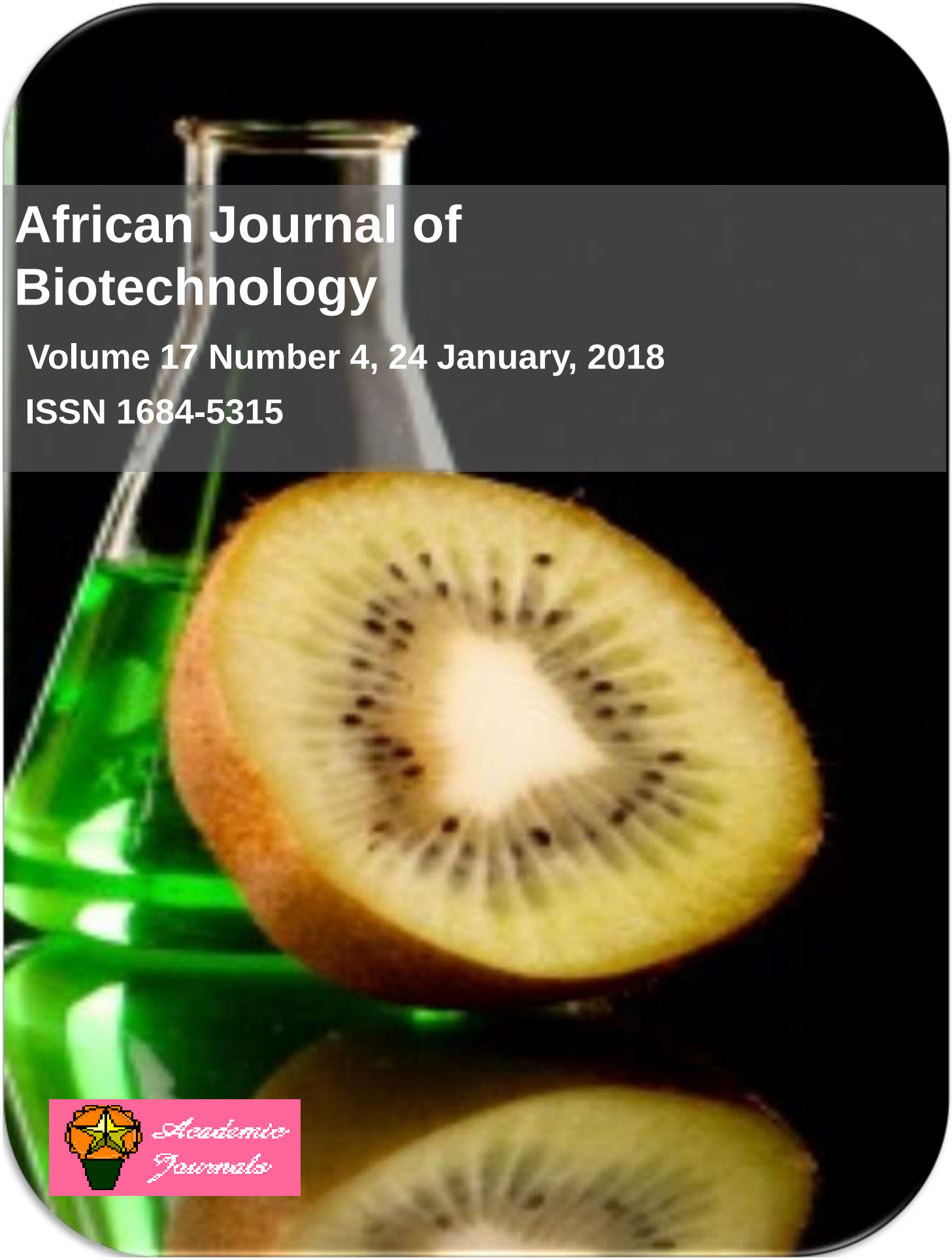

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Full Length Research Paper

Effect of antioxidants (taurine, cysteine, α-tocopherol) on liquid preserved Kolbroek boar semen characteristics

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Successful artificial insemination depends on maintaining longevity of semen in the reproductive tract, and semen characteristics among others. The study compared efficacy of Androhep extender supplemented with 75 mM taurine, 5 m M-cysteine, or 200 mg/l alpha-tocophero on liquid preservation of Kolbroek boar semen characteristics over 24 and 48 h after storage at 17°C. Total motility was evaluated using computer aided sperm analyzer (CASA), viability using SYBR/propidium iodidemembrane function using HOST test and lipid peroxidation using the MDA test. Statistical analysis was done using PROC GLM procedure of SAS (1996). Data were analyzed using analysis of variance (ANOVA). Untreated samples showed higher sperm quality in terms of membrane integrity, functionality and motility. All antioxidant treated spermatozoa did not show increased longevity (p>0.05) after 48 h of storage compared with untreated samples. Supplementation of Androhep extender with antioxidants did not improve sperm survivability of kolbroek liquid preserved semen. Therefore, the antioxidants were ineffective in improving longevity of Kolbroek liquid preserved semen after 48 h storage at 17°C. Further studies are required to find the effective antioxidant concentration to elicit sperm protection for Kolbroek boar semen liquid preservation.

Key words: Semen characteristics, longevity, lipid peroxidation.

INTRODUCTION

Kolbroek is a South African indigenous pig breed characterised by its unique gene pool. As such it is important to preserve the unique germplasm for conservation, research and future breeding purposes. The breed is extremely hardy and can survive under harsh conditions; hence this breed could be enrolled for cross breeding to improve adaptability traits in the swine meat industry. However, the knowledge on Kolbroek

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reproductive physiology is limited.

The effects of antioxidants are believed to have desirable effects in semen; however, the Kolbroek boar has shown some major differences in semen quality from the exotic landrace and large white boars therefore, there is need for further information on the sperm physiology of this pig breed.

Several extenders have been developed to preserve pig semen for long and short term storage. Kolbroek semen, has been extended with Beltsville thawing solution (BTS) and Kobidil used for short term storage; the latter performing better. Boar semen cooled to temperatures below 15°C, show an irreversible loss in sperm motility and metabolic activities (Orok et al., 2010). Thus for *in vitro* semen preservation, an extender should provide an environment that inhibits the formation of harmful reactive oxygen species (ROS) or lipid peroxide (Orok et al., 2010) and cold shock. Reactive oxygen species damages sperm membrane and DNA which in turn reduces the sperm's motility rate and ability to fuse with the oocyte which compromises the paternal genomic contribution to the embryo (Boonsorn et al., 2010). Preserving pig semen for short or prolonged period requires storage at low temperatures (15 to 18°C) however pig semen is known to be very sensitive to low temperature and is susceptible to lipid peroxidation during either liquid or cryopreserved (Basim et al., 2009) preservation forms. This is mainly due to the high continent of polyunsatured fatty acids (PUFAs) which is different from other species (Bresciani et al., 2012).

Some extenders have been shown to increase semen storage time for up to 3 days (Johnson et al., 2000), and even 5 to 7 days (Levis, 2000). Storing pig semen in cooled temperature around 5°C is a more cost effective alternative than liquid nitrogen preservation to help with increasing the use of artificial insemination in the pig industry. Therefore this study determines the efficacy of a long term pig semen extender supplemented with antioxidants (tocopherol, cysteine and taurine for liquid preservation of Kolbroek boar semen and possible increase in longevity during low temperature storage. The effectiveness of antioxidants in reducing the effect of reactive oxygen species damage is also evaluated at low temperature storage (17°C).

MATERIALS AND METHODS

Semen collection, processing and liquid storage

Semen was collected from three Kolbroek boars aged 9 to 12 months of proven good semen quality on four occasions using the hand gloved method (Roca et al., 2004). The sperm-rich fraction was collected using a thermo flask containing warm water (39°C) and a glass beaker covered with a gauze filter to separate the gel fraction from the sperm-rich fraction (Roca et al., 2004). Within an hour of collection, semen was transported to the laboratory for the evaluation. The semen was then diluted 1:1 (v/v) with isothermal Androhep extender supplemented with (i) 5 mM cysteine, (ii) 75 mM taurine, (iii) 200 µl α-tocopherol, and (iv) control/untreated to make four different treatments. After dilution, the four samples of the different treatments were then equilibrated at 17°C. Four replicates were used per treatment. Following equilibration at 0 h, then at 24 and 48 h the samples were evaluated using the swim-up (10 µL of semen was added to 500 µL of swim up media) method. Then 10 µL of each treated Kolbroek semen sample was placed in a 10 mL centrifuge tube containing 500 µL Bracket and Oliphant's sperm-wash media and co-incubated at 39°C for 5 min. After incubation, a drop (10 µL) of Kolbroek semen from each treatment was placed on a microscopic slide and evaluated microscopically for sperm progressive motility, viability and membrane permeability. Semen was analyzed just after collection, at 0 h, then at 24 and 48 h after collection in the different treatments of the antioxidants.

Sperm motility assessment using computer aided sperm analyser

Sperm motility characteristics were analyzed by the Sperm Class Analyzer (SCA, Masenya et al., 2012) (SCA® V.4.001) as follows; a drop (5 µL) of Kolbroek semen from each different sample treatment was placed on a microscopic slide and evaluated microscopically for sperm motility rate, with the aid of sperm class analyzer. Progressive motility was assessed and four replicates were evaluated per treatments.

Membrane permeability

The hyper osmotic swelling test (HOST) was performed to evaluate membrane permeability from each of the four replicates per treatment by employing the technique developed by Maxwell and Johnson (1997). Briefly, a 50 µl semen sample from each treatment and replicate was added and mixed with 1 ml of 150 mOsm/kg HOS diluent (7.35 g Na-citrate, and 13.51 g fructose, in 1 L of distilled water) and then incubated for 30 min at 37°C in 5% CO_2 incubator. The assessment of total sperm swelling and individual swelling patterns was recorded as coiled sperm tails which indicate good membrane functionality. A total of 200 spermatozoa were evaluated for coiled tails under a phase contrast microscope at 400x magnification. Four replicates were evaluated.

Viability

The percentages of viable sperm were evaluated from each of the four replicates per treatment using SYBR-14/propidium iodine (Fertilight, Sperm Viability Kit, Molecular Probes). Ten microliters (µl) of diluted spermatozoa from each treatment and replicate were mixed with 2.7 µl of the working solution containing SYBR-14 and 10 µl of propidium iodine. After incubation at 37°C for 20 min in the dark, a total of 200 spermatozoa were assessed (x400) using fluorescence microscopy (Carl Zeiss Inc., Axioskop 40, Oberkochen, Germany). The nuclei of spermatozoa with intact plasma membranes stained green with SYBR-14, while those with damaged membranes stained red with propidium iodine. The results were scored as the percentage of viable spermatozoa and non-viable (damaged and dead spermatozoa). Four replicates were evaluated.

Lipid peroxidation

Membrane lipid peroxidation was evaluated by the end point production of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) test (Esterbauer and Cheeseman, 1990). Briefly, diluted spermatozoa (250×10^6 cells in 1 mL of Androhep extender) from each treatment and replicate were mixed with 1.0

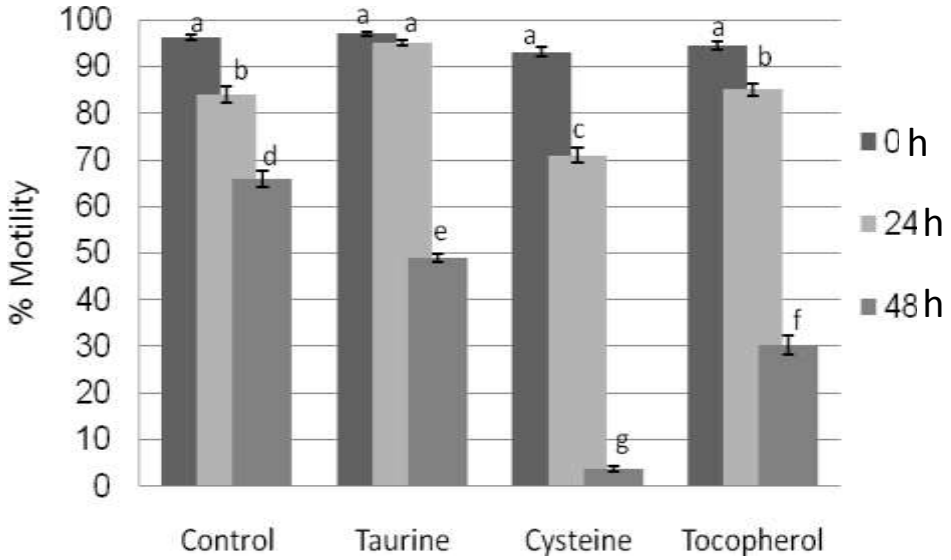

Figure 1. Progressive motility of liquid preserved Kolbroek boar semen equilibrated at 17˚C in Androhep extender supplemented with 5 mM cysteine, 75 mM taurine, 200 µl α-tocopherol, and control/untreated. Different letters between columns indicate significant differences between and across treatments (P<0.05).

mL of cold 20% (wt/vol) trichloroacetic acid (TCA) to precipitate protein. The precipitate was pelleted by centrifugation (1500 × *g* for 10 min), and 1.0 mL of the supernatant was incubated with 1.0 mL of 0.67% (wt/vol) TBA in a boiling water bath at 100°C for 10 min. After cooling to room temperature, the absorbance was determined by a spectrophotometer (UNICAM PU 8610 Kinetics spectrophotometer; Philips, Eindhoven, Holland) at 534 nm. The results were expressed as a simple concentration of MDA (nmol/ml). Four replicates were evaluated.

Statistical analysis

Statistical analysis was done using PROC GLM procedure of SAS (1996). Analysis of variance (ANOVA) was applied to determine the effects of antioxidants on motility, viability and membrane permeability of liquid preserved semen. Data on motility and viability was analyzed by general linear-models (time series with repeated measures) procedure. Treatment means were separated using Fisher's protected t-test least significant difference (LSD) at 5% level of significance (Snedecor and Cochran, 1980). Results are presented as means ± SEM.

RESULTS

Motility

Progressive motility decreased significantly ($p < 0.05$) over time from 0 to 48 h for all treatments, however the rate of total motility decline was higher for samples supplemented with antioxidants than control samples. With the exception of cysteine, taurine and tocopherol, treated samples showed higher total motility than untreated samples after 24 h storage. Control samples performed better than treated samples after 48 h storage. Taurine maintained the highest motility (95%) for 24 h with cysteine having the lowest motility of 71%. After 48 h, motility for all treatments declined below 50%, with taurine maintaining significantly ($p < 0.05$) higher motility of 49% and cysteine the lowest at 3% (Figure 1).

Membrane permeability

Membrane permeability decreased significantly ($p< 0.05$) with storage time. Cysteine was the least effective antioxidant in maintaining functional sperm membranes over 48 h. Taurine and tocopherol treated samples maintained membrane functionality better than untreated and cysteine treated samples. Rate of decline in membrane permeability was similar for untreated and treated samples from 24 to 48 h. Taurine (58%) treated samples showed the highest percentage of cells with functionaly active spermatozoa membrane and cysteine the least (30%) after 24 h, however there were no significant differences in efficacy of between taurine and tocopherol (Figure 2).

Viability

Viability was negatively affected by storage time. Untreated samples maintained viability better than treated samples over 48 h. Rate of decline in viable spermatozoa was higher for samples supplemented with antioxidants than untreated samples. Cysteine was the least effective antioxidant (Figure 3).

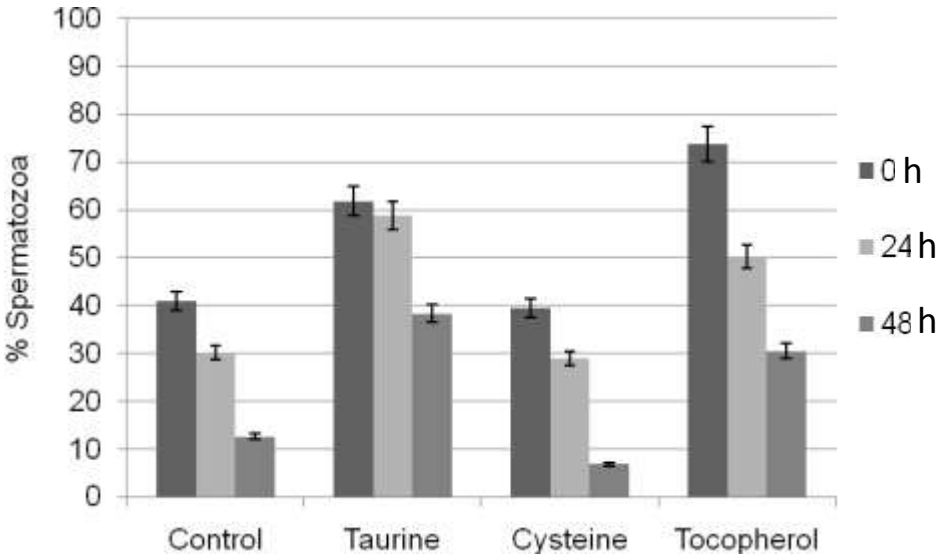

Figure 2. Membrane permeability of liquid preserved Kolbroek boar semen equilibrated at 17˚C in Androhep extender supplemented with 5 mM cysteine, 75 mM taurine, 200 µl α-tocopherol, and control/untreated.

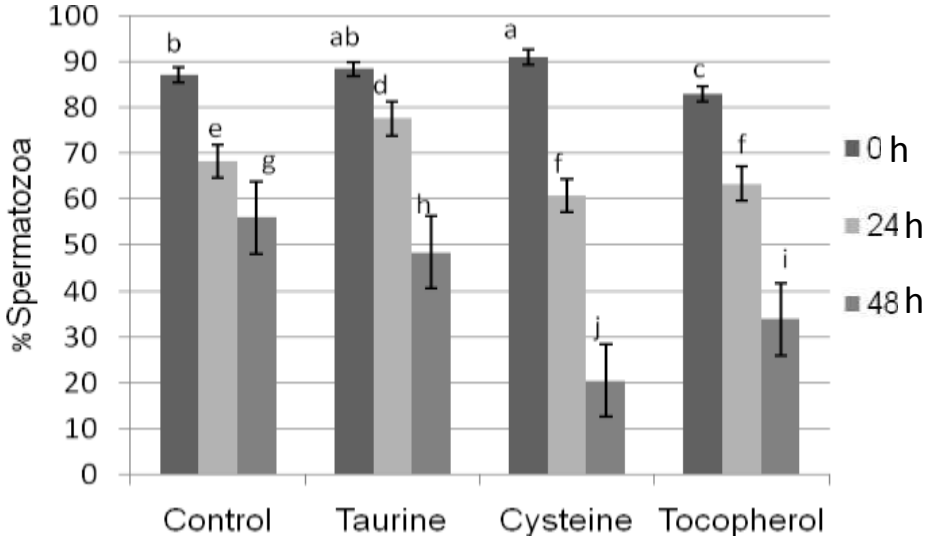

Figure 3. Viability of liquid preserved Kolbroek semen equilibrated at 17˚C in Androhep extender supplemented with 5 mM cysteine, 75 mM taurine, 200 µl α-tocopherol, and control/untreated. Different letters between columns indicates significant differences (P<0.05).

Lipid peroxidation

MDA concentration increased significantly with storage time indicating an increase in lipid peroxidation, with control samples showing the least antioxidant protection as expected addition of antioxidants to the extender had a positive impact on the decrease in the level of MDA during semen storage.

Cysteine was the most effective antioxidant in the maintenance of a constant MDA concentration from 0 to 48 h. Taurine had the highest concentration of MDA at 48 h, though it was not significantly different ($p > 0.05$) from tocopherol at 48 h (Figure 4).

DISCUSSION

Efficacy of antioxidants was lower than for untreated samples in for long term (48 h) lipid preservation of Kolbroek semen at 17°C. Control samples showed higher

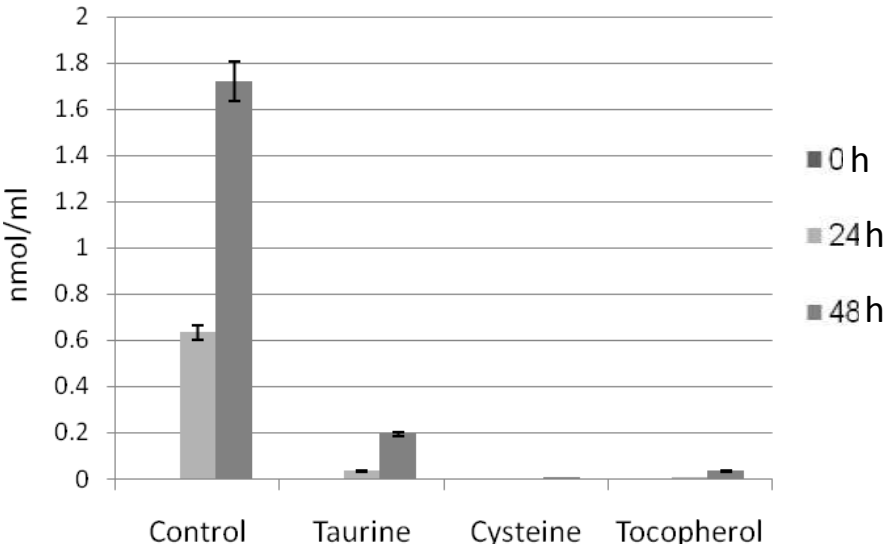

Figure 4. MDA concentration of liquid preserved kolbroek semen equilibrated at 17˚C in Androhep extender supplemented with 5 mM cysteine, 75 mM taurine, 200 µl α-tocopherol, and control/untreated.

motility, membrane functionality and integrity and general survivability over the 48 h storage. The study demonstrated that even though addition of antioxidants to extenders would decrease lipid peroxidation in general, the effective concentration and type of extenders is an important consideration when supplementing semen with antioxidants for protection against lipid peroxidation. Androhep extender (Vyt et al., 2004) which is a long term extender has been known to produce low survivability of semen compared to Beltsville thawing extender and Kobidil^{+} which are short term extenders for liquid preservation of pig semen. Long term extenders are designed to preserve semen for longer periods of time thus contain sufficient amounts of antioxidants to minimise and reduce the occurrence of reactive oxygen species (ROS) during the holding period. It was well established that ROS can damage the sperm plasma membrane and hence reduced the sperm motility rate (Guthrie and Welch, 2005; Chanapiwat et al., 2009). However, addition of further amounts of antioxidants in the concentration used in this study has no added advantage for low temperature short term storage of Kolbroek semen using a long term extender such as Androhep, and does not improve longevity of liquid preserved semen. In order to counter the effects of ROS damage, generally semen contains a complex redox system that combines the antioxidant potential of seminal plasma and the pro-oxidant potential of sperm. Enzymatic antioxidant defence mechanisms in seminal plasma and spermatozoa include superoxide dismutase, glutathione reductase, gluthathione peroxidase and catalase. Non-enzymatic antioxidants include reduced glutathione (GSH), urate, ascorbic acid, a-tocopherol, taurine, hypotaurine, carotenoids, and ubiquinones. The interplay of antioxidant and pro-oxidant mechanisms in semen determines the overall rate of lipid peroxidation in sperm (Ochsendorf et al., 1998).

Contrary with other studies, addition of antioxidants in the present concentrations in this study was ineffective in improving the longevity and survivability of the Kolbroek boar semen while the addition of antioxidants such as a α-tocopherol, butylated hydroxytoluene, superoxide dismutase, catalase, cysteine or glutathione in extenders to both cooled and frozen-thawed semen have been reported to improve the semen quality in boar (Funahashi and Sano, 2005; Breininger et al., 2005; Satorre et al., 2007), bull (Bilodeau et al., 2001a), turkey (Donoghue and Donoghue, 1997), stallion (Ball et al., 2001b) and ram (Uysal and Bucak, 2007).

Lipid peroxidation which has been shown to also occur in Kolbroek spermatozoa is described as a process under which oxidants such as free radicals or non-radical species attack lipids containing carbon-carbon double bond(s). Glycolipids, phospholipids (PLs), and cholesterol (Ch) are well-known targets of damaging and potentially lethal peroxidative modifiation (Ayala et al., 2002). Lipids can be oxidized by enzymes, for instance lipoxygenases, cyclooxygenases, and cytochrome P450. In response to membrane lipid peroxidation, and according to specific cellular metabolic circumstances and repair capacities, the cells may promote cell survival or induce cell death. Under physiological or low lipid peroxidation rates (subtoxic conditions), the cells stimulate their maintenance and survival through constitutive antioxidants defence systems or signalling pathways activation that upregulate antioxidants proteins resulting in an adaptive stress response. In contrast, under medium or high lipid peroxidation rates (toxic conditions)

the extent of oxidative damage overwhelms repair capacity, and the cells induce apoptosis or necrosis programmed cell death. Either process eventually leads to molecular cell damage which may facilitate development of various pathological states and accelerated aging (Kohen and Nyska, 2002) which may be the case in this study.

The decline in sperm characteristics during liquid preservation at 15-17°C has adverse effects on the structure of sperm cell membrane, with consequential in the failure of membrane functions due to the effects of ROS (Mazur et al., 2008). Cerolini et al. (2000) concluded that liquid storage of boar semen decreases sperm viability, motility and progressive motility due to the susceptibility of sperm cells to thermal, mechanical and osmotic stress during cooling of boar semen particularly at low temperature storage because pig semen is known to be very sensitive to low temperature.

Sperm function in pigs is altered rapidly during *in vitro* storage at 17 to 19°C in pigs, of which one of the supreme factors of failure is reactive oxygen species (Jang et al., 2006). ROS have twofold effects on sperm function, at low concentrations inducing sperm capacitation (Leclerc et al., 1997; Ford, 2004), hyperactivation (de Lamirande and Gagnon, 1993), acrosome integrity, and syngamy, while, in contrast, extreme amounts of ROS cause DNA damage (Aitken and Bennetts, 2005), inhibit syngamy, and reduce equine and porcine sperm motility of (Guthrie and Welch, 2007).

Semen preservation disrupts interactions between plasma lipids and proteins, which weakens membrane permeability and plasticity. This has been correlated to the processes of apoptotic cell death. Apoptosis is defined as programmed cell death (Said, 2004). Apoptotic cells have been correlated to lower motility, in Stallions, as a result of cellular changes, and also have a reduced longevity in the female reproductive tract of sows.

Even though this study shows little effect of antioxidants, these compounds are known to suppress the formation of ROS and protect spermatozoa against ROS damage (Sikka et al., 1995). Studies have demonstrated that seminal plasma also contains a number of enzymatic antioxidants such as superoxide dismutase (SOD, glutathione peroxidase/glutathione reductase (GPX/GRD) and catalase which protect the spermatozoa against lipid peroxidation. Antioxidants are believed to counteract the negative effects of lipid peroxidation hence posing a very positive chance of increasing motility, survivability and the functional membrane permeability (Satorre et al., 2007; Bilodeau et al., 2001b). Antioxidants such as L-cysteine for example play a role in the intracellular protective mechanism against oxidative stress as membrane stabiliser and capacitation inhibitor (Johnson et al., 2000).

L-cysteine can also improve survival time of semen and sperm chromatin structure in fresh chilled boar semen at 15°C (Szczesniak-Fabianczyk et al., 2003). Taurine is derived from cysteine, an amino acid which contains a thiol group. Taurine (2-aminoethanesulfonic acid) is the primary intracellular free-amino acid, which is normally present in most mammalian tissues (Chesney, 1985). Though it is not a component of any structural mammalian protein, taurine plays a variety of critical physiological roles including osmoregulation, cell propagation, viability and prevention of oxidant-induced injury in many tissues. The best known function of vitamin E or tocopherol as an antioxidant is the removal of free radicals that can initiate or propagate lipid oxidation. This action is due to its ability to interact faster with lipid peroxide radicals than they can do so with neighboring fatty acids or with membrane proteins. Tocopherol is anchored in the cell membrane through the isoprene chain, so that the chromatin ring that represents the active part of the molecule is on be on the surface of the structure of lipoproteins, in a position that would allow the interaction with the molecule and free radicals present in the cytoplasm. The tocopheroxil resulting radical reacts with another peroxyl radical, creating a stable product, tocopherol quinone. Thus, a single molecule of tocopherol is able to lead to the interruption of the two oxidation reactions (Ghiuru, 2013).

The current study concurs with that of Ball et al. (2001 b) that motility of boar spermatozoa was not significantly influenced by the addition of antioxidants especially α-tocopherol in particular thus the decline over time.

Increased levels of ROS have been linked with reduced motility. Many theories have been proposed to explain this correlation. One hypothesis is that hydrogen peroxide can diffuse into the cytoplasm via the cell membrane and inhibit the activity of enzymes such as glucose-6-phosphate dehydrogenase (G6PD) (Griveau et al., 1995). This enzyme controls the rate of flux of glucose via the hexose monophosphate shunt which then maintains the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). This molecule is used as a source of electrons by spermatozoa to fuel the generation of ROS by nicotinamide adenine dinucleotide phosphate oxidase, which is an enzyme system. Inhibition of G6PD leads to a decrease in the availability of NADPH and a concominant of oxidized glutathionine and reduced glutathionine. This reduces the defence mechanism of sperm antioxidants and can lead to membrane phospholipid peroxidation (Griveau et al., 1995) which results in a decline in viability as observed in the current study.

Contrary to this, Aguero et al. (1995) reported a positive effect of α-tocopherol on the motility of liquid preserved stallion semen. Andréa et al. (2008) also found a positive influence of cysteine on motility of refrigerated ram semen which is contrary to this study. According to this study, slight benefit of taurine was observed for less than 24 h of liquid storage. The valuable effects of taurine supplementation as an antioxidant in biological systems

have been credited to its ability to stabilize biomembranes (Wright et al., 1986), utilize reactive oxygen species (Wright et al., 1985) and reduce the production of lipid peroxidation end products (Huxtable, 1992).

Cysteine showed the least protection of cells over 48 h of liquid preservation. Three concentrations of the antioxidants were used in a preliminary study (results not published). 5 mM proved to produce better results, with cysteine though after 72 h all three concentrations had similar effect. This is in contrast to the results found by Aguero et al (1995). The probable reasons are not yet understood. Similar results were obtained with α-tocopherol.

This study shows that Kolbroek boar spermatozoa have unique responses. Preliminary studies (unpublished data), indicate that instead of Androhep extender being a long term extender as with other breeds, only managed to preserve the semen for 3 days, and yet the short term extender, BTS maintain survival for up to 12 h. Further research should be done on the composition of the poly unsaturated fatty acids and seminal plasma constituents of this breed in order to understand the physiology Kolbroek boar spermatozoa physiology in relation to other breeds. Also the bacterial contamination of the Kolbroek semen should be checked during further studies, in fact, as reported from other authors the presence of bacteria could lead to detrimental effects during storage at 15 to 17°C (Bresciani et al., 2014) It is important to evaluate also the different concentrations of the antioxidants and different storage temperatures to establish a more efficient protocol for Kolbroek boar spermatozoa liquid preservation. Taurine was the most effective antioxidant for liquid preservation of Kolbroek spermatozoa at 17°C. The most effective time to elicit antioxidant protection for Kolbroek spermatozoa liquid preservation was 24 h.

Conclusion

Supplementation of androhep extender with antioxidants (cysteine, taurine and tocopherol) was not effective for increasing longevity and survivability of liquid preservation of Kolbroek boar semen at 17°C. Semen characteristics were higher in the untreated sample than samples supplemented with the antioxidants during low temperature; lipid preservation of Kolbroek semen at 17°C. Control samples showed higher motility, membrane functionality and integrity and general survivability over the 48 h storage.

Conflicts of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Aguero A, Miragaya MH, Mora NG, Chaves MG, Beconi MT (1995). Effect of vitamin E addition on equine sperm preservation. Commun. Biol. 13:343-356.

Aitken RJ, Bennetts LE (2005). A comparative study of oxidative DNA damage in mammalian spermatozoa. Mol. Reprod. Dev.71:77-87.

Andréa HA, Stela Z, Coprean D, Dorina N (2008). Antioxidant additives effect on cytological parameters of refrigerated ram semen, Universitatea de Ştiinţe Agricole şi Medicină Veterinară Iaşi.

Ayala A, Muñoz MF, Argüelles S (2002). Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal, Oxidative Medicine and Cellular Longevity. Volume 2014, Article ID 360438, 31.

Ball B, Medina V, Gravance C, Baumber J (2001a). Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5ºC. Theriogenology 56:577-589.

Ball BA, Vo AT, Baumber J (2001b). Reactive oxygen species generation by equine spermatozoa. Am. J. Vet. Res. 62:5508-5515.

Basim J, Awda, Mackenzie-Bell M, Buhr MM (2009). Reactive Oxygen Species and Boar Sperm Function. Biol. Reprod. 81:553-561.

Bilodeau J F, Blanchette S, Gagnon C, Sirard MA (2001a). Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology 56:275-286.

Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C (2000b). Level of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing andthawing. Mol. Reprod. Dev. 55:282-288.

Boonsorn T, Kongbuntad W, Narkkong N, Aengwanich W (2010). Effects of catechin addition to extender on sperm quality and lipid peroxidation in boar semen. J. Agric. Environ. Sci. 7:283-288.

Breininger E, Beorlegui NB, O'Flaherty CM, Beconi MT (2005). Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. Theriogenology 63:2126-2135.

Cerolini S, Maldjian A, Surai P, Noble R (2000). Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Anim. Reprod. Sci. 58(1-2):99-111.

Chanapiwat P, Kaeoket K, Tummaruk P (2009). Effects of DHA-enriched hen egg yolk and Lcysteine supplementation on quality of cryopreserved boar semen. Asian J. Androl. 11:600-608.

Chesney RW (1985). Taurine: its biological role and clinical implications. Adv. Pediatr. 32:1-42.

De Lamirande E, Gagnon C (1993). A positive role of the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. Int. J. Androl. 16:21-25.

Donoghue AM, Donoghue DJ (1997). Effects of water and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage. Poult. Sci. 76:1440-1455.

Ford WC (2004) Regulation of sperm functions by reactive oxygen species. Hum. Reprod. Update 10:387-399.

Funahashi H, Sano T (2005). Select antioxidants improve the function of extended boar semen stored at 10 degrees C. Theriogenology 63:1605-1616.

Griveau JF, Dumont E, Renard P (1995). Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. J. Reprod. Fertil. 103:17-26.

Guthrie HD, Welch GR (2007). Use of fluorescence-activated flow cytometry to determine membrane lipid peroxidation during hypothermic liquid storage and freeze-thawing of viable boar sperm loaded with 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid. J. Anim. Sci. 85:1402-1411.

Guthrie HD, Welch GR (2005). Impact of storage prior to

cryopreservation on plasma membrane function and fertility of boar sperm. Theriogenoly 63:396-410.
Huxtable RJ (1992). Physiological actions of taurine. Physiol. Rev. 72(1):101-63.
Jang HY, Kong HS, Park CK, Oh DJ, Lee SG, Cheong HT , Kim JT, Lee J, Yang BK, Lee HK (2006) Effects of Taurine on Sperm Characteristics during In vitro Storage of Boar Semen. Asian Aust. J. Anim. Sci. 19(11):1561-1565.
Johnson LA, Weitze KF, Fiser P, Maxwell WMC (2000). Storage of boar semen. Anim. Reprod. Sci. 62:143-172.
Kohen R, Nyska A (2002). Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions, and Methods for Their Quantification. Toxicol. Pathol. 30(6):620-650.
Leclerc P, de Lamirande E, Gagnon C (1997). Regulation of protein tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free Radic. Biol. Med. 22:643-656.
Masenya MB, Mphaphathi ML, Mapeka MH, Munyai PH, Makhafola MB, Ramukhithi FV, Malusi PP, Umesiobi DO, Nedambale TL (2012). Comparative study on semen characteristics of Kolbroek and Large White boars following computer aided sperm analysis® (CASA). Afr. J. Biotechnol. 10:14223-14229.
Mazur P, Leibo SP, Seidel GE Jr (2008). Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. Biol. Reprod. 78:2-12.
Ochseedorf FR, Buhl R, Bästlein A, Beschmann H (1998). Glutathione in spermatozoa and seminal plasma of infertile men. Hum. Reprod. 13:353-359.
Orok EE, Essien A, Akpet SO, Ibom LA, Etop SC (2010). Mean sperm concentration and percent motility of extended porcine semen as affected by antibiotics from selected sources and storage time. Int. J. Curr. Res. 10:1-6.
Roca J, Gil MA, Hernandez M, Parrilla I, Vazquez JM, Martinez EA (2004). Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J. Androl. 25:397-405.
Said TM, Paasch U, Glander HJ, Agarwal A (2004). Role of caspases in male infertility. Hum. Reprod. Update 10:39-51.
Satorre MM, Breininger E, Beconi MT, Beorlegui NB (2007). Alpha-tocopherol modifies tyrosine phosphorylation and capacitation-like state of cryopreserved porcine sperm. Theriogenolgy 68:958-965.
Sikka SC, Rajasekaran M, Hellstrom WJG (1995). Role of oxidative stress and antioxidants in male infertility. J. Androl. 16:464-468
Szczesniak-Fabianczyk B, Bochenek M, Smorag Z, Silvestre MA (2003). Effects of antioxidants added to boar semen extender on the semen survival and sperm chromatin structure. Reprod. Biol. 3:81-87.
Uysal O, Bucak MN (2007). Effects of Oxidized Glutathione, Bovine Serum Albumin, Cysteine and Lycopene on the Quality of Frozen-Thawed Ram Semen. Acta Vet. Brno 76:383-390.
Vyt P, Maes D, Dejonckheere E, Castryck F, Soom AV (2004). Comparative study on five different commercial extenders for boar semen. Reprod. Domest. Anim. 39:8-12.
Wright CE, Tallan HH, Lin YY, Gaull GE (1986). Taurine: biological update. Ann. Rev. Biolchem. 55:427-453.
Wright CE, Lin TT, Syurman TA, Gaull GE (1985). Taurine scavenges oxidized chlorine in biological systems. In. Taurine: biological actions and clinical perspectives (Ed. S. S. Oja et al.), Alan. R. Liss. Inc., New York. pp. 137-147.

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Full Length Research Paper

Antioxidant and antikindling effect of *Tapinanthus globiferus* growing on *Ficus glumosa* in pentylenetetrazole induced kindled rats

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***Tapinanthus globiferus* growing on *Ficus glumosa* is a plant used locally for the treatment of epilepsy. It is found in West Africa on many tree crops. The present study aims to investigate the anti-kindling and antioxidant activity of the aqueous extract of *T. globiferus*. A total of 40 rats were divided into 4 groups (n=10). Groups 1 to 3 received 100, 200 and 400 mg/kg, respectively, of the extract orally, followed by 35 mg/kg of pentylenetetrazole (PTZ) *i.p* after an hour. Group 4 (control) was given 35 mg/kg of PTZ and normal saline and also observed for 30 min. This was repeated after every 48 h until all rats in the control group became fully kindled, that is, attained a racine score of 4 or 5 on three consecutive occasions. At the end of the experiment, the brain tissues of all rats were removed, homogenized and analyzed for antioxidant effect using lipid peroxidation, reduced glutathione, catalase, superoxide dismutase and uric acid tests. The extract was observed to significantly ($p < 0.001$) reduce the development of stage 5 kindling state as compared to the control group. The extract also significantly ($p < 0.05$) increased the activity of superoxide dismutase in the group treated with 400 mg/kg and also increased the activity of catalase in the 100 mg/kg treated group as compared to the control. The data obtained from this study suggests that the aqueous extract of *T. globiferus* growing on *F. glumosa* may possess bioactive compounds with antikindling and antioxidant effect and this may support its traditional use in the management of epilepsy.**

Key words: Antikindling, *Tapinanthus globiferus,* antioxidant, epilepsy, pentylenetetrazole rats.

INTRODUCTION

Epilepsy is a chronic disease affecting up to 1% of the population, making it second to stroke as one of the most common serious neurological disorders (Carl, 2006). It is a disorder in which the balance between cerebral excitability and inhibition is tipped towards uncontrolled excitability and characterized by recurrent unprovoked seizures (Gregory and Yehezkiel, 2001). The cause of most cases of epilepsy is unknown, although some

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people develop epilepsy as a result of brain injury, stroke, brain tumors, infection of the brain and birth defects (WHO, 2016). Therapy is symptomatic in that available drugs inhibit seizure but neither effective prophylaxis nor cure is available (McNamara, 2001).

Traditional systems of medicine are popular in developing countries and up to 80% of the population relies on traditional medicines or folk remedies for their primary health care need (Akerele, 1988). Several plants used for the treatment of epilepsy in different systems of traditional medicine have shown activity when tested in modern bioassays for the detection of anticonvulsant activity (Raza et al., 2003) and many such plants need to be scientifically investigated.

Chemical kindling is a phenomenon used for understanding the epileptogenic process involved in development of seizures and for studying molecules that have the potentials to prevent this process (Holmes, 2007). Kindling is a phenomenon ensuing from progressive intensity of convulsion activity due to repetitive administration of electrical or chemical sub-convulsive stimulators (Pavlova et al., 2004). If the stimulus causes generalized convulsion in experimental animal, it is accepted that kindling is completed and it is agreed that this abnormal excitable status remain permanent (Erdogan et al., 2006). Chemical kindling seizures induced with pentylenetetrazole (PTZ) are human absence epilepsy and myoclonic, generalized tonic-clonic and it is a model for drug resistance epilepsy (Ali et al., 2005).

The present study aims to investigate the antiepileptogenic potentials of TG used in parts of Sokoto and Kebbi states for the treatment of epilepsy. Through personal communication with traditional medicine men from these states, ethno-medical information on the use of the plant in the management of epilepsy was obtained.

MATERIALS AND METHODS

Plant collection and identification

The whole plant of *Tapinanthus globiferus* growing on *Ficus glumosa* (TG) was collected in Zuru Local Government Area, Kebbi State in the month of July, 2016. It was authenticated by an expert through a previously identified specimen of the plant deposited at the herbarium of the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria with a voucher specimen (UDUH/ANS/0076).

Preparation of the extract

The whole plant was dried at room temperature and pulverized into powder using pestle and mortar. The powder was accurately weighed and subjected to aqueous extraction by maceration and subsequent filtering. The filtrate was thereafter dried in an oven at 45°C. After sufficient drying, the extract was scraped from the evaporating dish and weighed to ascertain the yield.

Experimental animals

Adult rats of both sexes weighing between 83 and 200 g were obtained from the Animal house facility of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The animals were maintained in a well-ventilated room under ambient laboratory conditions of temperature and humidity and were fed on commercial feed and water throughout the course of the experiment. All experimental protocols were approved by the University Animal Ethics Committee. Forty rats were grouped into 4 groups of 10 rats (n=10). Group 1 served as the control which received normal saline, while groups 2 to 4 received TG extract at doses of 100, 200 and 400 mg/kg, respectively.

Phytochemical screening

The dried aqueous extract of the whole plant of TG was subjected to phytochemical screening tests for the detection of various constituents using the method described by Sofowora (2008) and Trease and Evans (2002).

Acute toxicity study

The acute toxicity studies of TG have been previously reported by Abubakar et al. (2016) to be ≥ 5000 mg/kg po.

Antikindling study

Pentylenetetrazole (PTZ) induced seizure in rats

The method described by Goddard et al. (1969) and Racine (1972) was employed for this study. 40 rats were divided into 4 groups, n=10 rats. Groups 2 to 4 were given 100, 200 and 400 mg/kg, respectively, of the extract orally, followed by 35 mg/kg of PTZ i.p after an hour and were observed for 30 min. The first group was treated with 35 mg/kg of PTZ and normal saline and was observed for 30 min. Seizure scores 30 min after each PTZ injections were defined as follows: (1) Phase 0: no response; Phase 1: ear and facial twitching; Phase 2: myoclonic body jerk; Phase 3: clonic forelimb convulsion; Phase 4: generalized clonic convulsions, turning onto one side position; Phase 5: generalized clonic-tonic convulsions (or death within 30 min).

Antioxidant activities of *T. globiferus* growing *on F. glumosa*

Determination of lipid peroxidation in PTZ-kindled rat brain homogenates

Lipid peroxidation was estimated by the measurement of malondialdehyde (MDA) levels, and its level was determined spectrophotometrically by use of thiobarbituric acid reactive substances (TBARS) method previously described (Ohkawa et al., 1979).

Twenty four hours after the 35 mg/kg PTZ challenge, the kindled rats were sacrificed by decapitation and the whole brain was removed and homogenized (100 mg/ml) in ice-cold 0.1 M phosphate buffer (7) (Ohkawa et al., 1979). One hundred and fifty microliter of the supernatant was diluted to 500 µl with double deionized water. 250 µl of 1.34% thiobarbituric acid were added to all the tubes, followed by addition of equal volume of 40% trichloroacetic acid. The mixture was shaken and incubated for 30 min in hot boiling water bath with a temperature > 90°C .Tubes were allowed to cool to room temperature and the intensity of the pink-coloured complex formed was measured at 532 nm in a spectrophotometer using 0 concentration as blank.

Determination of reduced glutathione in the brain of kindled rats

The method described by Patterson and Lazarow (1955) was employed. The principle is based on the fact that glutathione reacts with an excess of alloxan to produce a substance with an absorption peak at 350 nm.

Enzymatic assay for catalase in the brain of PTZ kindled rats

The method of Beers and Sizer (1952) was employed. The principle involves:

$2H_2O_2$ catalase $2H_2O + O_2$

The disappearance of peroxide is followed spectrophotometrically at 240 nm. One unit is equal to one µmole of hydrogen peroxide decomposed per minute under specified conditions of 25°C.

Enzymatic assay of superoxide dismutase in the brain of PTZ kindled rats

The method of Zou et al. (1986) was adopted, 6 tubes were arranged in rows of threes, to the first row, 0.10 ml of buffer, 0.83 ml of distilled water and 0.05 ml of sample brain homogenate were pipetted, respectively. To the second row, 0.15 ml, 0.83 and nil samples were pipetted, respectively. The test tubes were incubated at 25°C for 10 min, and then transferred into a cuvette and 0.02 ml pyrogallols were added. The content was mixed thoroughly by inversion and the increase in absorbance was measured at 430 nm using the maximum linear rate for both test and blank.

Determination of uric acid in PTZ-kindled rat brain

Uric acid level was determined using REDOX kit, following the protocol described by the manufacturer.

Determination of gain in body weights

The weight gain of the rats was assessed weekly and recorded until the day of sacrifice, the initial weight prior to initiation of PTZ administration was noted and compared with the final weight post-kindling.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) and percentages. Data analysis was performed using Graph Pad Prism statistical software (version 6.0). Comparison between groups was made using analysis of variance (ANOVA) and Kruskal Wallis test where necessary. When a statistically significant difference was obtained, a *post hoc* Dunnets test or Dunns test was performed for multiple comparisons depending on the nature of data. Values of $p < 0.05$ were considered significant.

RESULTS

Percentage yield

Percentage yield of the extract was found to be 8.5% w/w.

Table 1. Phytochemical screening of *Tapinanthus globiferus* whole plant.

Constituent	Results
Carbohydrate	+
Tannins	+
Alkaloid	+
Saponnin	+
Flavonoid	+
Antraquinones	+
Proteins	+
Glycosides	+
Steroid	+
Triterpenes	-

+= Present; - =absent.

Phytochemical screening

The phytochemical screening of *T. globiferus* growing on *F. glumosa* revealed the presence of carbohydrates, tannins, alkaloids, saponins, flavonoids, anthraquinones, proteins, glycosides and steroids with absence of triterpenes as shown in Table 1.

Effect of *T. globiferus* growing on *F. glumosa* in pentylentetrazole induced kindled rats

The result of forty eight hourly treatments of rats with 35 mg/kg of pentylentetrazole on alternate days showed a progressive increase in convulsion response. The control group and 100 mg/kg extract treated group reached the Racine score of 5.0 on the 10^{th} day. In the 200 and 400 mg/kg treated group, the extract of *T. globiferus* significantly ($p < 0.001$) decreased the development of kindling, and the maximum seizure score was 1 in the 400 mg/kg treated group and 3 in the 200 mg/kg treated group respectively, as shown in Figure 1.

Effect of *T. globiferus* on weight gain in pentylentetrazole induced kindled rats

The results of 48 hourly administration of aqueous extract of *T. globiferus* to rats indicate a dose dependent increase in the mean weight gain which is statistically insignificantly ($p > 0.05$) as shown in Table 2.

Effect of *T. globiferus* aqueous extract on antioxidant enzymes in the brain of pentylenetetrazole induced kindled rats

The extract at a dose of 400 mg/kg significantly ($p < 0.05$) increased the activity of superoxide dismutase (Figure 5).

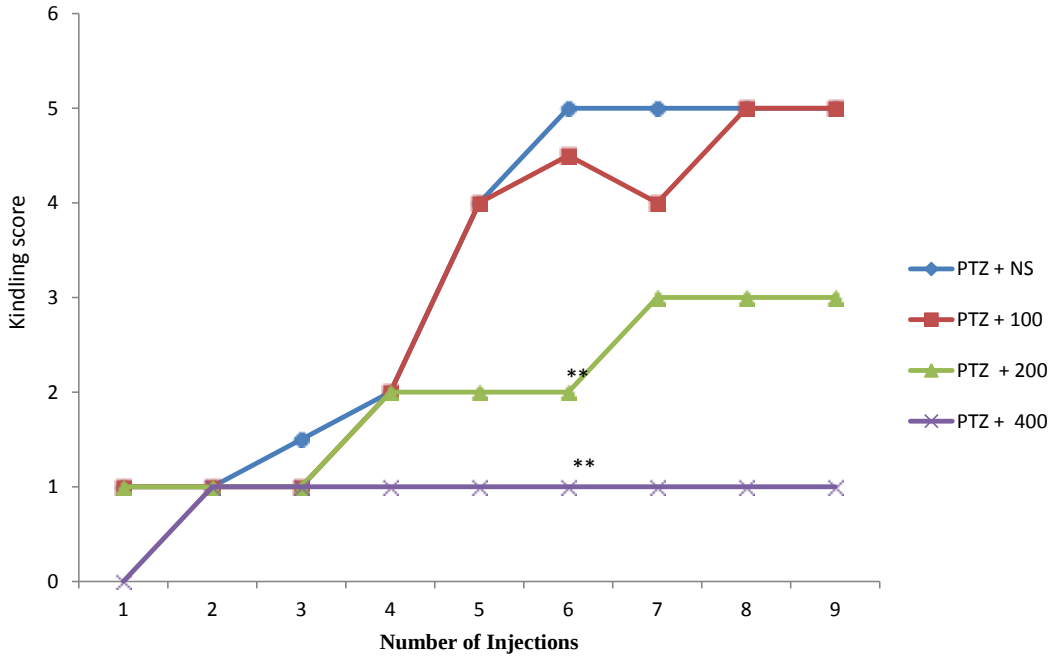

Figure 1. Effect of *T. globiferus* on pentylentetrazole induced kindled rats. Result presented as median scores; n=10; ** p < 0.001 as compared to the control group. Kruskal-Wallis test, followed by Dunn's *post hoc*.

Table 2. Effect of *Tapinanthus globiferus* on weight gain in pentylentetrazole induced kindled rats.

Treatment (mg/kg)	Initial weight (g)	Final weight (g)	Weight gain (g)
Control	132.0 ± 10.5	177.4 ± 11.2	45.4 ± 0.7
100	126.5 ± 10.6	179.4 ± 9.7	52.9 ± 0.9
200	128.7 ± 8.9	182.1 ± 10.2	53.4 ± 1.3
400	131.4 ± 9.0	180.9 ± 11.4	49.5 ± 2.4

Values are expressed as mean ± SEM, n=10.

In addition, there was a significant ($p < 0.05$) increase in the activity of catalase (Figure 6) in the 100 mg/kg treated group relative to the control. Figures 2, 3 and 4 show the effects of the plant extract on the activities of reduced glutathione, lipid peroxidation and uric acid, respectively, in the brain of control and pentylenetetrazole kindled rats. There is an insignificant increase ($p > 0.05$) in the activities of these enzymes.

DISCUSSION

Phytochemical screening of *T. globiferus* revealed the presence of constituents such as alkaloids, saponins, tannins, glycosides, protein, steroids and flavonoids. Alkaloids have been reported to function as amoebicides, expectorant, anesthetic, analgesic and anti-helminthes (Fabricant and Farnsworth, 2001). Mistletoe alkaloids are sequestered by the parasite from the host tree (Gill and Onyibe, 1990). Flavonoids and tannins detected in the *T. globiferus* growing on *F. glumosa* leaves have been implicated to play significant roles in the metabolism of lipids (Abolaji et al., 2007). Anticonvulsant effect of saponins and flavonoids has been reported by Shibata (2001).

The effect of the aqueous extracts (200 and 400 mg/kg) of *T. globiferus* on pentylenetetrazole induced kindling indicated antiepileptogenic activity. The ability of this compound to abolish seizure induced by pentylenetetrazole may be due to the extracts ability to antagonize one of the several mechanisms of pentylentetrazole action which includes: interaction with γ-aminobutyric acid (GABA) neurotransmission, central noradrenergic activity and/or blockage of glutamatergic neurotransmission mediated by N-methyl D-aspartate (NMDA) (Khan et al., 2013).

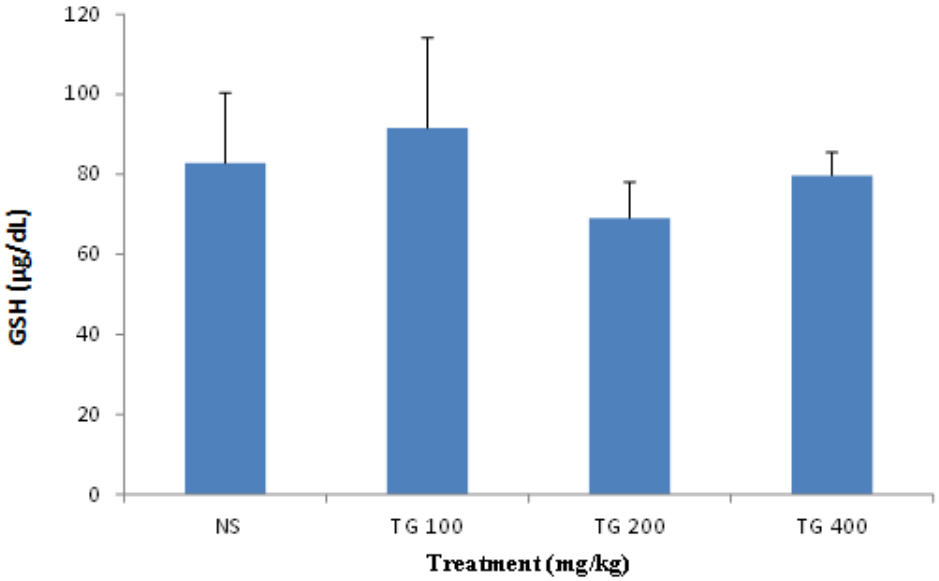

Figure 2. Effect of *T. globiferus* aqueous extract on reduced glutathione (GSH), n=10, TG = *T. globiferus,* NS= normal saline.

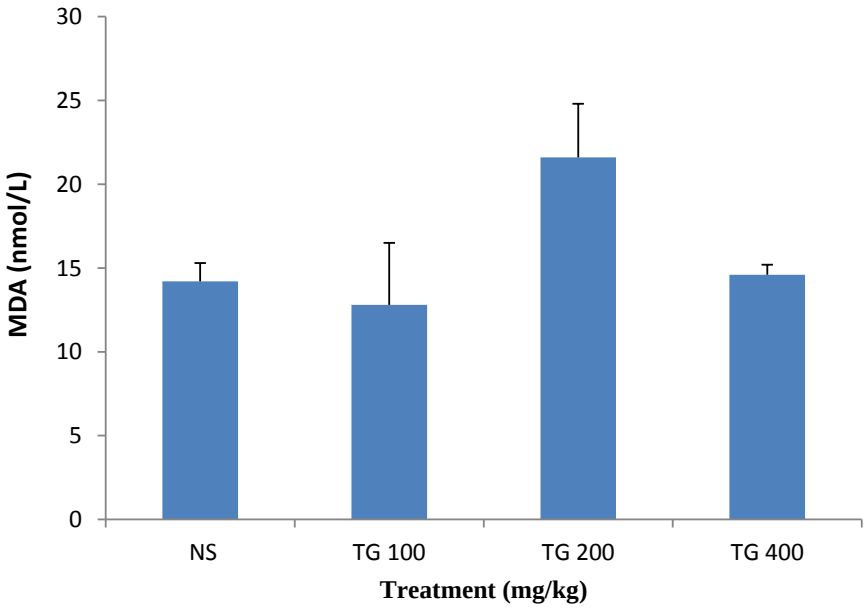

Figure 3. Effect of *T. globiferus* aqueous extract on lipid peroxidation, n=10, TG = *T. globiferus,* NS= normal saline.

Pentylentetrazole induced kindling resulted in mortalities in the control and 100 mg/kg treated group and this may be due to little or no protection received from the extract as compared to the group that received 200 and 400 mg/kg of the extract in which there was no mortality. *T. globiferus* shows antioxidant effect in the superoxide dismutase and catalase assay, this corroborates the findings of Adekunle et al. (2012) that the leave extract may serve as a good antioxidant due to its high iron chelating capacity. The relationship between antioxidant and anticonvulsant activities has been previously reported (Patrick, 2011; Rahmati et al., 2013). Epilepsy is accompanied by reversible convulsions which induce production of reactive oxygen species (ROS) in

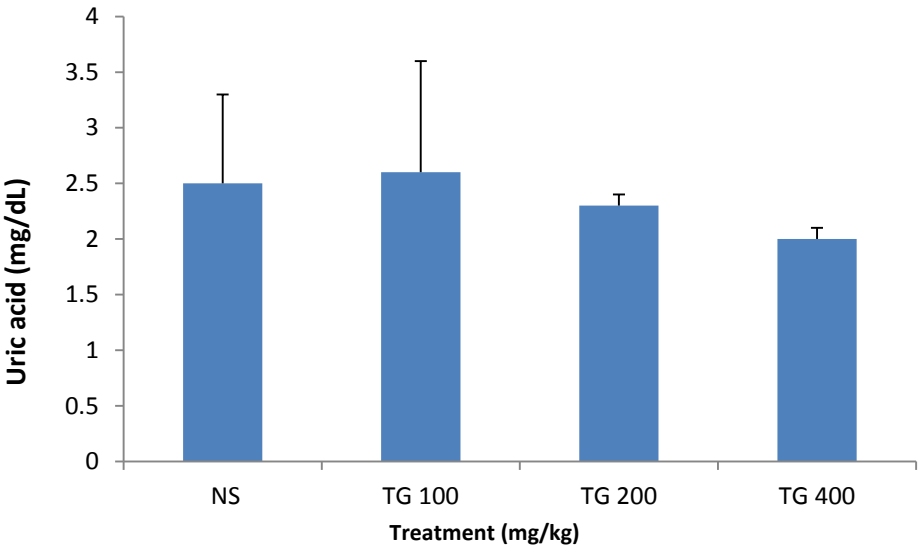

Figure 4. Effect of *T. globiferus* aqueous extract on uric acid. n=10, TG = *Tapinanthus globiferus,* NS= normal saline.

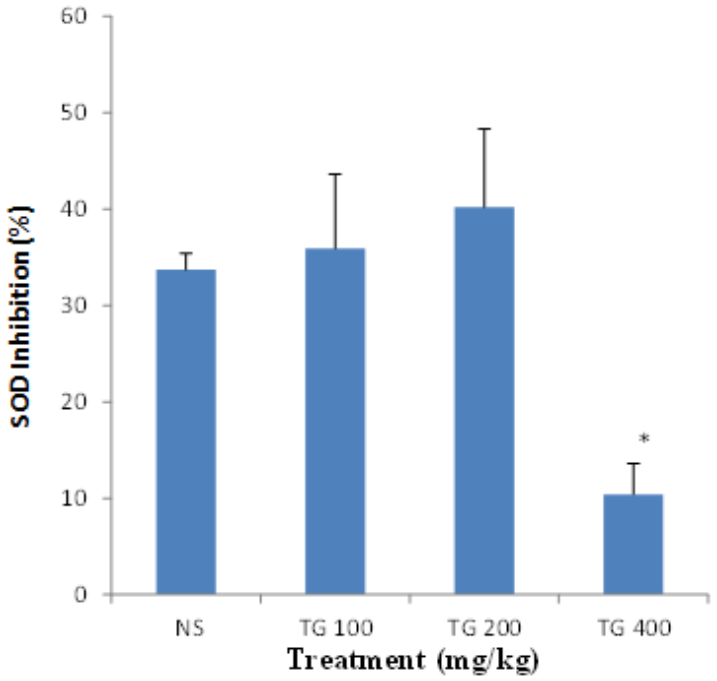

Figure 5. Effect of *T. globiferus* aqueous extract on superoxide dismutase (SOD), n=10, *p < 0.05 as compared to the control group. One way ANOVA followed by Dunnett's *post hoc*. TG = *T. globiferus,* NS= normal saline.

the brain, and these free radicals are reported to mediate convulsion development (Rahmati et al., 2013). Free radical causes lipid peroxidation at polyunsaturated sites on biological membranes and tissue injury which leads to cell membrane destruction and cell dysfunction (Gupta and Sherma, 1999). The ability of the plant to abolish epileptogenesis may be due to its antikindling and antioxidant activity. The aqueous extract of *T. globiferus*

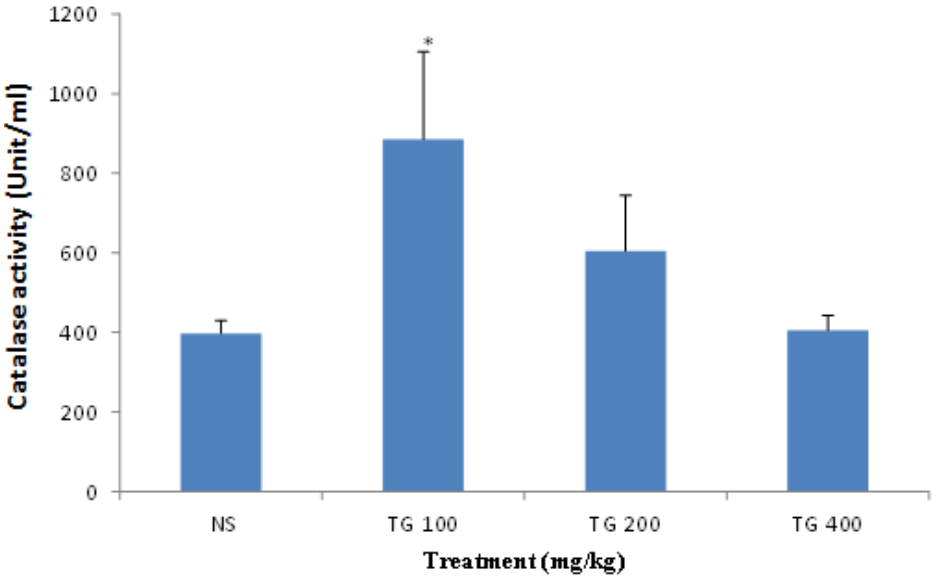

Figure 6. Effect of *T. globiferus* aqueous extract on catalase enzyme. n=10, *p < 0.05 as compared to the control group. One way ANOVA followed by Dunnett's *post hoc*. TG = *T. globiferus,* NS= normal saline.

did not cause significant changes in the weight of rats after the eighteenth day of treatment ($p > 0.05$). This effect may be due to the absence of noxious compounds in the aqueous extract of *T. globiferus* which may cause anorexia. Recent study on the antikindling effect of plants showed a significant reduction in weight in the extract of untreated group post-kindling (Abubakar, 2017).

Conclusion

The result of this study suggests that the aqueous extract of *T. globiferus* growing on *F. glumosa* may possess bioactive compounds with antioxidant and antikindling activity and this may support its traditional use in the treatment of epilepsy.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

REFERENCES

Abolaji AO, Adebayo AH, Odesanmi OS (2007). Effect of ethanolic extract of Parinari pilyandra (Rosaceae) on serum lipid profile and some electrolytes in pregnant rabbits. Res. J. Med. Plant 1:121-127

Abubakar K (2017). Anticonvulsant and chronic toxicity studies of the methanol stem bark extract and fractions of *Pseudocedrela kotschyi* in laboratory animals Unpublished PhD thesis

Abubakar K, Adebisi IM, Ugwah-Oguejiofor JC, Idris GO, Idris B, Mshelia HE (2016). Phytochemical screening and anticonvulsant activity of the residual aqueous fraction of *Tapinanthus globiferus* growing on *Ficus glumosa* Herb. Med. 2(2):1-6.

Adekunle AS, Aline AB, Afolabi OK, Rocha JB (2012). Determination of Free Phenolic acids, Flavonoid contents and Antioxidant Capacity of Ethanolic Extracts Obtained from leaves of Mistletoe (*Tapinanthus globiferus*). Asian J. Pharm. Clin. Res. 5(3):36-41.

Akerele O (1988). Medicinal plants and primary health care: an agenda for action. Fitoterapia 59(5):355-363.

Ali A, Ahmad FJ, Pillai KK, Vohora D (2005). Amiloride protects against pentylenetetrazole-induced kindling in mice. Br. J. Pharmacol. 145(7):880-884.

Beers RF, Sizer IW (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195(1):133-140.

Carl ES (2006). A review of selected clinical syndromes and advances in basic science. J. Cereb. Blood Flow Metab. 26:983-04.

Erdogan F, Küçük A, Gölgeli A (2006). The assessment of the features of seizures and EEG in Pentylenetetrazole-induced kindling. J. Neurol. Sci. 23(2):84-92.

Fabricant DS, Farnsworth NR (2001). The Value of Plants Used in Traditional Medicine for Drug Discovery. In. Environment Health Perspective, New York: McGraw-Hill Companies Inc. 109(1):69-75.

Gill LS, Onyibe HI (1990). Mistletoes on rubber trees in Nigeria. Haustorium 23:1-2.

Goddard GV, McIntyre DC, Leech CK (1969). A permanent change in brain function resulting from daily electrical stimulation. Exp. Neurol. 25:295-330.

Gregory LH, Yehezkiel Ben-A (2001): The Neurobiology and Consequences of Epilepsy in the Developing Brain. Paediatr. Res. 49(3):320-325.

Gupta YK, Sharma M (1999). Oxidative stress in neurological disorders. New Delhi: Society of Biosciences/jamai Hamdard/Asiatech Publ.

Holmes GL (2007). Animal model studies application to human patients. Neurology 69(24 Suppl 3):S28-S32.

Khan F, Musa Y, Yaro AH, Yahuza N (2013). Anticonvulsant Activity of Methanol Leaf Extract of *Commiphora kerstingii* Engl. Basic Sci. Med. 2(1):9-13.

McNamara JO (2001). Drugs effective in the therapy of the epilepsies. In. Hardman JG, Limbird LE, Goodman Gilman A, 10th ed. Goodman and Gilman's the pharmacological basis of therapeutics. New York: McGraw-Hill. pp. 521-547.

Ohkawa H, Ohishi N, Yagi K (1979). Assay for lipid peroxides in animal

tissues by thiobarbituric acid reaction. Anal. Biochem. 95(2):351-358.
Patrick A (2011). Anticonvulsant and related neuropharmacological effects of a hydro-ethanolic whole plant extract of *Syndrella nodiflora* (L. Gaertin, Fam: Astraceae). Unpublished PhD research thesis.
Patterson JW, Lazarow A (1955). Determination of glutathione. In. Glick D, (Ed). Methods of Biochemical Analysis. Interscience 2:259-279.
Pavlova TV, Yakovlev AA, Stepanichev MY, Mendzheritskii AM, Gulyaeva NV (2004). Pentylenetetrazole kindling induces acti-vation of caspase-3 in the rat brain. Neurosci. Behav. Physiol. 34(1):45-47.
Racine RJ (1972). Modification of seizure activity by electrical stimulation: II. Motor seizure. Electroencephalogr. Clin. Neurophysiol. 32(3):281-294.
Rahmati B, Khalili M, Roghani M, Ahghari P. (2013). Antiepileptogenic and antioxidant effect of *Lavandula officinalis* aerial part extract against pentylentetrazole induced kindling in male mice. J. Ethnopharmacol. 148(6):152-157.
Raza M, Shaheen F, Choudhary M I, Rahman A.U, Sombati S, Suria A, DeLorenzo RJ (2003). Anticonvulsant effect of FS-1 subfraction isolated from roots of Delphinim denudatum on hippocampal pyramidal neurons. Phytother. Res. 17(1):38-43.
Shibata S (2001). Chemistry and Cancer preventing Activities of Ginseng saponins and some related triterpenoid compounds. J. Korean Med. Sci. 16(supplement):S28-S37.
Sofowora A (2008). Medicinal plants and traditional medicine in Africa. 3rd Edition, Spectrum Books Limited Ibadan, Nigeria. Pp. 199-202.
Trease GE, Evans MC (2002). Phytochemistry In: Textbook of Pharmacognosy. Fourth edition. WB Sanders Company Ltd. London, UK. pp. 224-343.
WHO (2016). Epilepsy Fact Sheet. Retrieved 4 March, 2016. www.who.int/mediacentre/infographic/mental-health/epilepsy/en.
Zou, GL, Gui XF, Zhong XL, Zhu YF (1986). Improvements in pyrogallol autoxidation method for the determination of SOD activity. Prog. Biochem. Biophys. 4:71-73.

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Full Length Research Paper

Optimized production of phytase by solid-state fermentation using triticale as substrate and source of inducer

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This study was carried out to evaluate the process of phytase production by *Aspergillus niger* in solid-state fermentation (SSF) using triticale waste. A waste that currently has no use was reported for this biotechnological process, and is of high impact due to the null use. The process was carried out using an additive free medium, supplemented with only one nitrogen source. Under these conditions, phytase activity of 7.45 U/g dry substrate (DS) was obtained. The process was optimized using different additives such as dextrose, lactose, Tween 80 and potassium chloride. For fermentation maximization, two experimental designs were used: 1) Plackett-Burman design (PBD) and 2) the Box-Behnken design (BBD). PBD was used to evaluate the effect of related variables on the production of phytase, as well as their level of significance in the process, while BBD was used for optimal conditions determination. The process was conducted with Petri dishes and a maximum enzyme activity of 25.8 IU/g DS was obtained. Subsequently, SSF was carried out in a tray to increase the amount of fermented substrate and phytase activity of 23.63 IU/g DS was obtained. The results of this study suggest a minimal decrease (8.4%) in enzyme production with scaling.

Key words: Triticale, solid-state fermentation, phytase, *Aspergillus niger*, optimization, statistical experimental design.

INTRODUCTION

Solid state fermentation (SSF) is a technique which has been known for hundreds of years. It is defined as a metabolic process by which organisms grow on a solid matrix, in the absence or near absence of free water (Díaz et al., 2007; Pal and Khanum, 2010). Substrates used in SSF process are generally not soluble in water, and are integrated as carbon source, vitamins and minerals; therefore, they must have enough moisture to

support the growth and metabolic activities of microorganisms (Ali and Zulkali, 2011; Graminha et al., 2008). In recent times, SSF has been used with great success in the production of a large number of metabolites of interest, including antibiotics, surfactants, organic acids, aromatic compounds, pesticides and many enzymes. In this process, the incorporation of agroindustrial wastes mainly as substrates which are beneficial, is needed for the production of metabolites of interest, and is accessible at no cost (Bhavsar et al., 2010; Díaz et al., 2007; Haefner et al., 2005; Pandey et al., 2001).

Phytases are hydrolytic enzymes, phosphatases type, and belong to the subfamily of histidine acid phosphatases. They are responsible for catalyzing the hydrolysis of phytate phosphor mono ester bonds (salts myo-inositol hexakisphosphate) or myo-inositol 1,2,3,4,5,6-hexakisdihidrogeno phosphate (phytic acid) producing derivatives, such as tetra, tri, di and inositol monophosphate and inorganic phosphate (Pi) (Shivanna and Venkateswaran, 2014; Albarracín et al., 2013; Bilgiçli et al., 2006). This enzyme is mainly applied in the animal feed industry, where it is used as supplement in feeds of non-ruminant animals (such as pigs, chickens, turkeys, etc.). This is because phytic acid is the largest reservoir of phosphorus in plant, with 60 to 80% bound to different compounds. In monogastric animals, phosphorus is largely unavailable in phytic acid due to the absence of phytase in their digestive system. This leads to the elimination of phytic acid via stool, which consequently results in soil pollution and eutrophication of water by phosphates (Fei et al., 2013; Ma et al., 2011; Vats et al., 2009).

Phytase is an enzyme that liberates the Pi present in phytic acid and makes it available for digestion in animals (Vats et al., 2009; Velayudhan et al., 2015). When used as a supplement, it has been shown to reduce the Pi in manure by about 33%, which ensures a third less environmental pollution and improvement of animal nutrition. The main limitation to the use of this enzyme with high nutritional and environmental interest is the high market price and, in some cases, lower levels of production (Haefner et al., 2005; Romero et al., 2009).

This enzyme is produced by submerged fermentation (SF) or by solid substrate fermentation (SSF). Studies have shown that the best alternative method used for the production of this enzyme is through the application of SSF (Haefner et al., 2005; Marlida et al., 2010; te-Biesebeke et al., 2002). Phytase can be directly produced in SSF by filamentous fungi using some agro-industrial residues as substrate to add value to the process, and supplemented with various additives to favor its production (Pal and Khanum, 2010). The rigorous processes of SSF were recently described to optimize the production process and, at the same time, to maximize yield (Bhavsar et al., 2010; Saad et al., 2011). The classification of this enzyme as Generally Recognized as Safe (GRAS), provides a large field of study because of the great benefits it brings to phytase, and the relative ease with which it is produced. This is intended to intensify both the search for new microorganisms, as well as abundant waste utilization, with little or no market value. This provides an environment that is similar to the native environment of microorganism for the development and production of adequate metabolites.

Triticale (Triticosecale Wittmack) is a synthetic crop species developed by crossing wheat and rye. Its peculiar name originates from the union of the scientific names of the two genera involved: wheat (Triticum) and rye (Secale). The peculiarity of triticale is that it combines the features of rye genome, such as hardiness, disease and environmental tolerance, with the high yield potential and grain quality of wheat (Cantale et al., 2016). These features make it a suitable crop for any environment with little or no susceptibility to biotic stress. Thus, this saves the cost of production as compared to other crops. This resulted in high grain yield, low loss and high biomass production. Besides its great culture qualities, it has been found to be beneficial in the recovery of contaminated soil, and in promoting the growth of carbon-fixing microorganisms (Borneo et al., 2016; Giunta et al., 2015). Therefore, despite being a new crop, it has been shown to have huge benefits as compared to other crops. The growing interest in its production can be attributed to the increasing amount of soil intended for cultivation, resulting in an increase in unused materials, thereby causing waste (Jondreville et al., 2007). This provides a great opportunity for the incorporation of these residues in the biotechnological production processes of industrial metabolites of high interest. Thus, one of the main objectives of this study was to evaluate a sufficiently produced waste in the state of Coahuila, Mexico, which has been previously studied as livestock feed. Therefore, the objective of this study was to use triticale agro-industrial waste as a substrate for the production of phytase by fermentation in solid medium and maximize production levels by improving the nutritional parameters of the medium.

MATERIALS AND METHODS

Fungus and inoculum preparation

The *Aspergillus niger* 7A-1 strain was provided by the

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Nanobioscience Group, University Autonomy of Coahuila, Saltillo, México. It belongs to a group of strains isolated from the semi-desert of Coahuila, México. Prior to the commencement of the study, the strain was grown and maintained on potato dextrose agar (PDA) slants at 28 ± 1°C, to obtain the inoculum. After seven days of fungal growth, spores were collected with 0.1% Tween 80 solution. Their concentration was adjusted to1 x 10^6 spores/mL (Bhavsar et al., 2010).

Designs of SSF culture medium

Substrates used for the production of phytase enzyme were obtained from the experimental fields of the Agrarian Autonomy University, "Antonio Narro". The substrate was washed with distilled water to remove soil and impurities and dried at 60°C. It was separated in spike and stems, then ground to obtain a particle size of approximately 0.3 mm and stored separately in sealed bags until use. Three different mixtures were made between spike and stems of the residues, which is defined in Table 1.

SSF in Petri dish

Five grams (5 g) of dry substrate (DS) was placed in a Petri dish and sterilized at 121°C for 20 min. After cooling, the substrate was supplemented with 3 mL solution containing NH_4NO_3 4% (w/v), previously sterilized. The substrate was inoculated with 0.5 mL spore suspension of fungus at 1×10^6 spores/mL (Bhavsar et al., 2010). The Petri dish was incubated for 5 days at 28 ± 1°C. The moisture content of the inoculated substrate was approximately 60%. All the experiments were carried out in triplicate and the average values were taken.

SSF scaling tray

500 g of selected blend previously dried, was placed in an aluminum tray (300 mm x 150 mm x 60 mm) with a layer thickness of 3 cm. Thereafter, it was inoculated with 50 mL of spore solution, and the moisture was adjusted to 60% with the mentioned optimized medium. The trays were covered with plastic wrap, incubated at 28 ± 1°C for 5 days with 99% relative humidity in a chamber. They were ventilated twice every day for five minutes. The trays were sampled every 24 h under aseptic conditions to obtain a representative sample of 2 g. The substrate and the solution (with adjusted moisture) were sterilized at 121°C for 20 min before use.

Enzyme activity assay

The phytase activity was determined in crude extract (SSF) obtained from sample with distilled water (5 mL/g sample) and stirred for 1 h (200 rpm at 25 ± 1°C). The suspension was centrifuged (10,000 g for 10 min) and the supernatant (crude extract) was stored at 4°C until further utilization (Mittal et al., 2011).

Phytase activity was determined by measuring the Pi released from sodium phytate solution (Harland and Hraland, 1980; Monteiro et al., 2015). The reaction mixture consisted of 1 mL of 0.1 M $MgSO_4*7H_2O$, 2.4 mL of 6.82 mM phytic acid and 0.6 mL of appropriately diluted crude enzyme solution. Solutions of $MgSO_4*7H_2O$ and phytic acid were prepared with 0.2 M sodium acetate buffer (pH 5.15). Subsequently, the reactants were incubated at 55°C for 60 min, and the reaction was stopped by adding 0.5 mL of 10% trichloroacetic acid. Thereafter, 1 mL of distilled water and 2.4 mL of Taussky-Schorr reagent (10 mL of 10 N H_2SO_4, 1 g of $(NH_4)Mo_7O_{24}*4H_2O$ and 5 g of $FeSO_4*7H_2O$ graduated to 100 mL distilled water) were added to generate a blue chromophore (Harland and Hraland, 1980). The content was mixed for 30 min and then the absorbance was determined at 660 nm. Measured values were correlated with a standard curve that was constructed using monopotassium phosphate. One unit of phytase activity was defined as the amount of enzyme that released 1 µmol of phosphate per minute under assay conditions. All the enzyme activity analyses were performed in triplicate.

Table 1. Mixtures used for the preselection of SSF medium for the production of phytases by *A. niger* 7A-1, based on their content of spike and straw.

Mixture	Spike (%)	Straw (%)
M1	25	75
M2	50	50
M3	75	25

Other analytical determinations

Protein concentration was determined by the Bradford method using bovine serum albumin as the standard at 0 to 20 µg/mL (Bradford, 1976).

Experimental design to optimize the medium selected and statistical analysis

The aim of applying this experimental design was to identify components that promote the high production of phytase enzyme. The Plackett-Burman (PBD) experimental design facilitates the analysis of multiple independent variables in a process, and help in demonstrating their significance (Liu and Tang, 2010). Several studies have identified seven variables that are effective in the production process (Bhavsar et al., 2010; Mittal et al., 2011). Two reference values for each variable were used, the higher level was denoted by "+" and the lower by "-" (Table 2).

The experiments were carried out in Petri dishes with 5 g of medium selected, to which was added a medium constituting seven additives, two carbon sources, one surfactant, three mineral sources and one phosphoric acid for pH adjustment (Table 2) under the conditions described above at constant 60% moisture content. The SSF was carried out under the conditions described above. The effect level of each selected variables was determined by the difference between the average responses. The significance level of each variable was determined by Pareto charts, and statistical analyses of the data were carried out using analysis of variance (ANOVA) and Student´s *t*-test mean comparison test ($p<0.05$) (Statistica® 7, Statsoft, 217 Tulsa, OK, USA). The Box-Behnken design (BBD) was used to investigate the relationship between variables of medium components, and to optimize their concentrations for the best yield of phytase enzyme production in SSF, using *A. niger* (Bogar et al., 2003; Mittal et al., 2011). In experiments of the present study, four factors at three levels of variations were defined (Table 5). Subsequently, results were obtained through the application of analysis of variance (ANOVA). To design the experiments, analysis of variance and process maximization were done using Statistical Package 7 for Windows, for regression analysis of the experimental data obtained. To determine the significance of regression coefficients, a *t*-test was applied. All experimental designs were randomized. The experiments were carried out in triplicate in Petri dishes, and the mean values were recorded.

Table 2. The Plackett–Burman design for physical variables and nutrients in phytase production by *A. niger* 7A-1.

Code	Variables	Levels	
		High (+)	Low (-)
A	Dextrose % (w/w)	8	0.8
B	Lactose % (w/w)	8	0.8
C	Tween 80 % (w/w)	0.6	0.3
D	KCl (mg/g DS)	0.5	0.25
E	K_2PO_4 (mg/g DS)	1	0.5
F	EDTA (mg/g DS)	1	0.5
G	Phosphoric acid (mL/kg DS)	2	1

RESULTS AND DISCUSSION

Selection of the mixture appropriate for phytase production

The mixtures analyzed for the production of phytase enzyme yielded highly significant results. In the M3, the amount of enzyme produced was the lowest. 3.67 IU/g was probably due to the high concentration of nutrients contributed by spike, thus avoiding the need for the microorganism to synthesize the enzyme of interest. Also, concentrations of spike and forage in equal parts, (M2) only produced 5.76 IU/g DS, which can be attributed to the fact that the fermenting medium has a larger compaction and this makes the aeration processes difficult. For this reason, M1 was selected for obesity studies. With this composition, it was possible to obtain 7.45 IU/g DS of phytase by having a concentration of nutrients that allows the induction of phytase and maintains an adequate aeration atmosphere allowing the proper development of the microorganism.

Screening of significant nutrients using the Plackett-Burman design (PBD) for SSF by *A. niger*

In the basal medium selected, 7.45 IU/g DS was produced in the fifth day of fermentation (Figure 1). The PBD for eight trials with two levels of concentrations were carried out to evaluate the significance of the seven medium components shown in Table 3 (Zhang et al., 2006). This table also shows the phytase activity obtained in each of the experiments, wherein an extensive difference between each of the treatments was observed. Between 10 and 40%, an increase in the levels of phytase activity (Table 3) was obtained. This variation demonstrates the importance of medium supplementation in achieving higher productivity. These results are similar to those reported by Bhavsar et al. (2010) where *A. niger* managed to raise phytase enzyme production by 34% using waste wheat and various additives.

To differentiate the important process variables in SSF and their degree of influence, Pareto chart was used (Figure 1). The chart shows bars with a length proportional to the value of the effect on the studied process. The bars are displayed in accordance with the size of effects, with the largest effects on top. The diagram for any individual effect allows an evaluation of the probability of finding the observed random effect (Meena et al., 2013). Figure 1 shows a vertical line at the critical *t*-value α of 0.50, and the effect for which the bar is smaller than the critical *t*-value is considered variable, having no significant effect on the studied process.

Table 4 shows the total sum of squares and respective percentage contribution for each variable. The results are also shown in Figure 1. It is also shown that the variable E, F and G, have a negligible contribution. D (KCl) showed greater significance in process, similar to that reported by Bhavsar et al. (2010) who used *A. niger* and wheat based medium supplemented with KCl among others, to show the importance of this compound using PBD.

The highest phytase activity obtained was 12.5 IU/g DS. This result was obtained in the basal production medium containing only the solid substrate and 40 g/kg $(NH_4)_2NO_3$ as source of nitrogen after 120 h of incubation. It was found to be 40% higher than the initial 7.45 IU/g DS.

The coefficient of regression obtained was $R^2 = 0.9314$ and it was shown that the model used for analyzing the data is significant. This indicates that the model explains 93% of the variability in data. Four medium components (Table 3) were identified as significant like variables for phytase production by BBD and after model optimization, BBD was used. According to Singh and Satyanarayana (2011), carbon source plays an important role in the production of enzymes, in particular, the use of simple sugars as an important variable for phytase production. Besides that, it directly influences the regulation of moisture levels by interacting with solutes present in the SSF medium (Mittal et al., 2011).

Table 5 shows the value used in maximization. Table 6

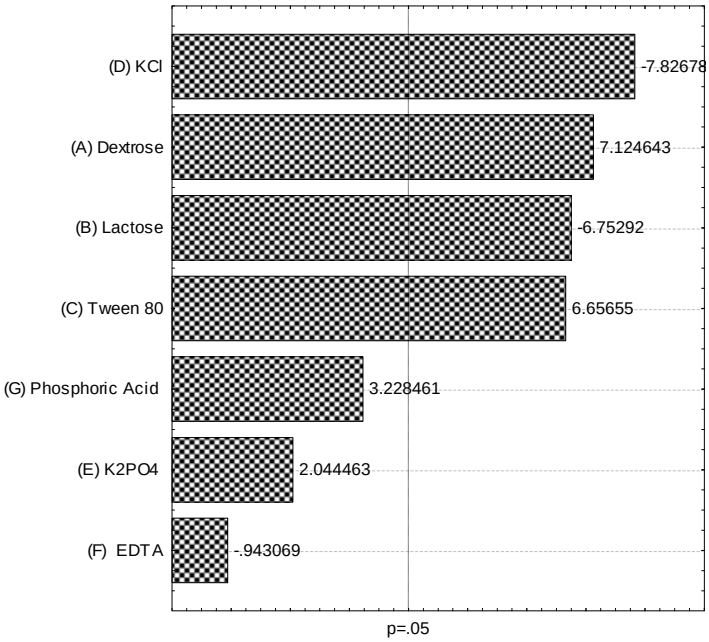

Figure 1. Pareto bar charts for the estimation of effects (absolute values) of the independent variables (factors) present in the SSF for phytase production by *A. niger* 7A-1. The extent of the bars along the vertical dotted line (p = 0.05) represents the significance dimension.

Table 3. Variables analyzed in the PBD design and its response as a function of phytase activity yields detected for each variable for improvement in yields of phytase enzyme produced by *A. niger* 7A-1.

Trial	Variables							Phytase activity
	A	B	C	D	E	F	G	IU/g DS
1	-1	-1	-1	1	1	1	-1	8.43
2	1	-1	-1	-1	-1	1	1	12.13
3	-1	1	-1	-1	1	-1	1	8.10
4	1	1	-1	1	-1	-1	-1	7.54
5	-1	-1	1	1	-1	-1	1	8.60
6	1	-1	1	-1	1	-1	-1	12.50
7	-1	1	1	-1	-1	1	-1	10.35
8	1	1	1	1	1	1	1	11.37

Table 4. Percent of contribution and ANOVA of each variable and its effect on phytase production in triticale waste by *A. niger* 7A-1.

Variable code	Sum of squares	Contribution (%)
A*	17.85	1.78
B*	16.03	1.60
C*	15.58	1.55
D*	21.54	2.15
E	1.47	0.014
F	0.31	0.003
G	3.66	0.036

*Highly significant.

Table 5. Levels used for the BBD design of each variable that showed significant difference on phytase production by *A. niger* 7A-1 in SSF.

Code	Variables	Levels		
		-1	0	+1
A	Dextrose %(w/w)	10.0	12.0	14.0
B	Lactose %(w/w)	0.2	0.4	0.6
C	Tween 80 % (v/w)	1.0	1.5	2.0
D	KCl mg/g DS	0.15	0.20	0.25

Table 6. BBD matrix and result of experiments by phytase production with *A. niger* 7A-1 using triticale waste.

Trial number	Factor level				Response of phytase activity (IU/g DS)	
	A	B	C	D	Experimental	Predictive
1	-1	-1	-1	-1	11.03	11.15
2	-1	-1	0	1	11.43	11.60
3	-1	-1	1	0	13.03	13.14
4	-1	0	-1	1	10.78	11.05
5	-1	0	0	0	12.16	12.36
6	-1	0	1	-1	12.98	13.00
7	-1	1	-1	0	13.63	13.93
8	-1	1	0	-1	14.20	14.34
9	-1	1	1	1	12.78	15.02
10	0	-1	-1	1	11.44	11.70
11	0	-1	0	0	13.15	13.00
12	0	-1	1	-1	13.54	13.65
13	0	0	-1	0	12.57	12.46
14	0	0	0	-1	12.66	12.87
15	0	0	1	1	13.36	13.55
16	0	1	-1	-1	14.25	14.44
17	0	1	0	1	14.59	14.88
18	0	1	1	0	16.67	16.42
19	1	-1	-1	0	13.78	13.58
20	1	-1	0	-1	13.79	13.99
21	1	-1	1	1	15.35	14.67
22	1	0	-1	-1	13.25	13.44
23	1	0	0	1	13.68	13.89
24	1	0	1	0	15.57	15.43
25	1	1	-1	1	15.69	15.46
26	1	1	0	0	15.13	16.76
27	1	1	1	-1	17.46	17.41

shows the design matrix of four significant variables in coded levels and reported experimentally, the phytase activity obtained. The trial number 27 had the highest phytase activity with 17.41 IU/g DS. The experimental results obtained for phytase production were analyzed using backward elimination regression.

The regression coefficients, t- and P-values, are present in Table 7. Following the analysis, the quality of the obtained model was evaluated on various criteria. One is their correlation coefficient R^2, which was 0.9561 for phytase production. In terms of percentage, this indicates that 95.61% corresponds to variability in the aforementioned model. The value of the correlation coefficient predicted for phytase production was 0.9383, which shows a strong agreement between the experimental and predicted values of phytase production. The coordinates of the maximum point found are: A=1.2, B=1.2, C=1.2 and D=-0.2, corresponding to the optimal supplementation levels of dextrose with 16.8 g, lactose 0.48 g, Tween 80 1 mL and KCl 0.2 g for each 100 g of triticale. In further experiments, triticale was supplemented with these optimized levels of additives. The results obtained in the present study showed a strong correlation agreement between predicted and experimental response.

Phytase production under maximized fermentation conditions was studied for 144 h. Figures 2 and 3 shows the observed difference between the optimized and un-

Table 7. Results of regression analysis of BBD in phytase production by *A. niger* 7A-1 in SSF with triticale waste. Coefficient of determination, R^2=0.95.

Source	Sum of squares	*t*-value	*P-value*
Intercept	13.0368	50.7015	0.000000
A	0.9754	5.0896	0.000005
B	1.0667	5.0808	0.000005
C	1.0412	6.04741	0.000000
D	-0.1215	-1.0028	0.320390
AB	0.2497	0.2746	0.784628
AC	-0.0663	-0.7297	0.468679
AD	0.3112	3.4333	0.001188
BC	-0.1040	-0.9914	0.325891
BD	-0.4980	-0.4750	0.636655
CD	-0.2271	-2.1640	0.034900
A^2	0.1219	0.3673	0.714767
B^2	1.0565	5.0325	0.000000
C^2	0.2810	1.3387	0.186271
D^2	-0.5950	-2.8343	0.006447

Table 8. Comparison of phytase production by other fungal strains under SSF.

Micro-organism	Production Technique	Substrate	Phytase activity (IU/g DS)	References
Aspergillus ficcum	SSF	Wheat bran	15	Bogar et al. (2003)
Aspergillus niveus	SSF	Wheat bran	3.4	El Gindy et al. (2009)
Mucor Racemosus	SSF	Groundnut oil cake	24.3	Roopesh et al. (2006)
Rhizopus oligosporus	SSF	Coconut oil cake	30.1	Ramachandran et al. (2005)
Rhizopus oryzae	SSF	Coconut oil cake	27.6	Ramachandran et al. (2005)
Aspergillus niger	SSF	Triticale wasted	25.8	Present work

optimized models. The maximized medium showed a phytase production of 7.45 IU/g DS, whereas maximization studies gave phytase production of 25.80 IU/g DS on the 5th day of fermentation. These results showed that phytase activity increased by 3.4 times in the maximized medium experiment.

The maximized results showed a productivity of 4,300 IU/kg/day. The results of productivity obtained in this study are highly competitive as compared to other previous studies. For example, Krishna and Nokes (2001) obtained a productivity of 4,667 IU/kg/day with *A. niger*. In their study, it was shown that fungi of the genus *Aspergillus* are the best producers of phytase (Bhavsar et al., 2013; Soni et al., 2010). Table 7 shows the results of other researches on the production of phytase with various agroindustrial residues. These data confirmed the competitiveness of the microorganism used in the present investigation against other microorganisms used for the production of phytase. Also, the data highlight the importance of triticale waste used in this research, because, the use of this waste in the production of enzymes is yet to be reported. The results showed that this residue is of value as compared to other highly studied agro-industrial residues, such as wheat bran or canola cake, in the production of phytase and other hydrolases (Table 8).

Estimation of phytase production by SSF

Given these results and considering that the requirements of phytase are 400 IU/kg of food. The crude extract of 15.5 kg of triticale was fermented to treat a metric ton (MT) of feed. An equivalent amount is needed to maintain 10 pigs per month (considering that a pig consumes between 3 and 4 pounds of feed per day). Definitely, it is highly profitable, taking into account that the cost of fermentation of substrate needed to treat a metric ton is US$ 28.48. Finding products to market reaches US$ 300 with only half prepared for deal in metric ton.

To confirm these results, an encouraging image was presented indicating the pilot production level. Products rich in phytase enzyme also contain some other hydrolytic enzymes that favor digestion in non-ruminants. This was reported by Bogar et al. (2003) who found alpha amylase, xylanase and others. The supplementation of these enzymes together with phytase contributes to a

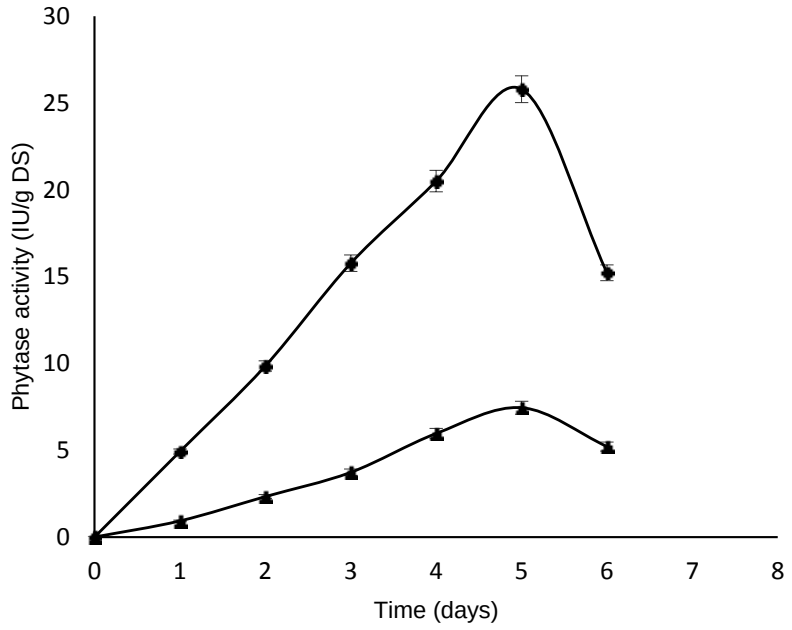

Figure 2. Kinetics of phytase production in SSF by *A. niger* 7A-1 under optimized conditions (filled squares) and without optimization (filled triangles).

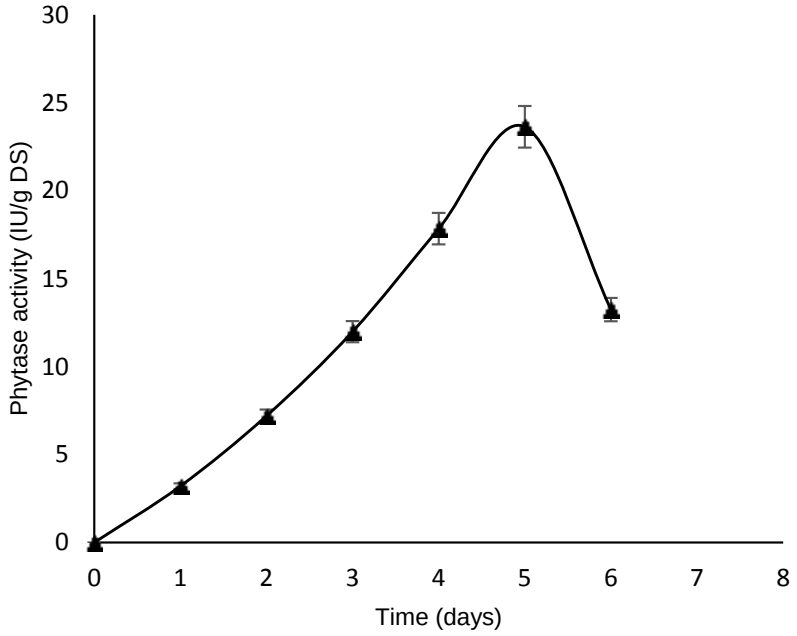

Figure 3. Kinetics of 6 days SSF by *A. niger* 7A-1 in a tray. Fermentation conditions: 500 g triticale dried on a tray (300 × 150 × 60 mm) under the conditions of optimization and initial moisture of 60% and 28±1°C.

decrease in the viscosity of food during digestion, amount of loose droppings, increases or mass gain, feed conversion and egg production in the case of poultry (Morales et al., 2011; Romero et al., 2009).

Up-scaling production of phytase enzyme by SSF in trays

SSF was performed in stainless steel stationary trays.

The procedure was scaled-up from 5 g waste triticale in Petri dish to 500 g waste triticale in trays: 300 mm × 150 mm × 60 mm. By scaling up from Petri dishes to stationary trays, the activities of 25.8 IU/g DS were reproducibly obtained. These results are therefore encouraging for maximization under pilot scale conditions. The scale up showed an 8.4% reduction in activity obtained in the Petri dish, which can be attributed to the enlargement process in the model.

Conclusion

Based on analysis of the results of this research, it can be concluded that the use of triticale as waste for the production of phytase, is highly feasible both from an economic point of view, as well as performance. The results obtained are highly significant and can lead to further investigation due to the importance of phytase in industrial animal feed. Both *A. niger* 7A-1 and triticale residue used showed high efficiency in the process of maximizing the production of phytase with minimum nutritional requirements. Keeping highly affordable process and increasing production is of great interest at the industrial production level. It should be noted that the total SSF process is a sustainable process which demonstrates the importance of using the null value of this residue in the production of metabolites. The test level scale showed a high feasibility of applying this waste for industrial phytase enzyme production while maintaining high yields, efficiency and low processing requirements cost. However, the results obtained in this study are of great importance, since the use of triticale as waste in the production of phytase showed high competitiveness against waste used industrially for commercial phytase production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Albarracín M, González RJ, Drago SR (2013). Effect of soaking process on nutrient bioaccessibility and phytic acid content of brown rice cultivar. LWT-Food Sci. Technol. 53:76-80.

Ali HKQ, Zulkali MMD (2011). Design Aspects of Bioreactors for Solid-State Fermentation. Chem. Biochem. Eng. Quarterl 25:255-266.

Bhavsar K, Buddhiwant P, Soni SK, Depan D, Sarkar S, Khire JM (2013). Phytase isozymes from *Aspergillus niger* NCIM 563 under solid state fermentation: Biochemical characterization and their correlation with submerged phytases. Process Biochem. 48:1618-1625.

Bhavsar K, Kumar VR, Khire JM (2010). High level phytase production by *Aspergillus niger* NCIM 563 in solid state culture: response surface optimization, up-scaling, and its partial characterization. J. Ind. Microbiol. Biotechnol. 38:1407-1417.

Bilgiçli N, Elgün A, Türker S (2006). Effects of various phytase sources on phytic acid content, mineral extractability and protein digestibility of tarhana. Food Chem. 98:329-337.

Bogar B, Szakacs G, Linden JC, Pandey A, Tengerdy RP (2003). Optimization of phytase production by solid substrate fermentation. J. Ind. Microbiol. Biotechnol. 30:183-190.

Borneo R, Alba N, Aguirre A (2016). New fi lms based on triticale fl our: Properties and effects of storage time. J. Cereal Sci. 68: 82-87.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-54.

Cantale C, Petrazzuolo F, Correnti A, Farneti A, Felici F, Latini A, Galeffi P (2016). Triticale for Bioenergy Production. Agric. Agric. Sci. Procedia 8:609-616.

Díaz AB, Caro I, de Ory I, Blandino A (2007). Evaluation of the conditions for the extraction of hydrolitic enzymes obtained by solid state fermentation from grape pomace. Enzyme Microb. Technol. 41:302-306.

Fei B, Xu H, Zhang F, Li X, Ma S, Cao YY, Xie J, Qiao D, Cao YY (2013). Relationship between Escherichia coli AppA phytase's thermostability and salt bridges. J. Biosci. Bioeng. 115:623-627.

Giunta F, Cabigliera A, Virdis A, Motzo R (2015). Field Crops Research Dual-purpose use affects phenology of triticale. F. Crop. Res. 183:111-116.

Graminha EBN, Gonçalves AZL, Pirota RDPB, Balsalobre MAA, Da Silva R, Gomes E (2008). Enzyme production by solid-state fermentation: Application to animal nutrition. Anim. Feed Sci. Technol. 144:1-22.

Haefner S, Knietsch A, Scholten E, Braun J, Lohscheidt M, Zelder O (2005). Biotechnological production and applications of phytases. Appl. Microbiol. Biotechnol. 68:588-597.

Harland BF, Hraland J (1980). Fermentative reduction of Phytate in Rye, White , and Whole Wheat Breads. Cereal Chem. 57:226-229.

Jondreville C, Genthon C, Bouguennec A, Carre B, Nys Y (2007). Characterisation of European varieties of triticale with special emphasis on the ability of plant phytase to improve phytate phosphorus availability to chickens. Br. Poult. Sci. 48:678-689.

Krishna C, Nokes SEEE (2001). Predicting vegetative inoculum performance to maximize phytase production in solid-state fermentation using response surface methodology. J. Ind. Microbiol. Biotechnol. 26:161-70.

Liu RS, Tang YJ (2010). Tuber melanosporum fermentation medium optimization by Plackett-Burman design coupled with Draper-Lin small composite design and desirability function. Bioresour. Technol. 101: 3139-3146.

Ma XF, Tudor S, Butler T, Ge Y, Xi Y, Bouton J, Harrison M, Wang ZY (2011). Transgenic expression of phytase and acid phosphatase genes in alfalfa (Medicago sativa) leads to improved phosphate uptake in natural soils. Mol. Breed. 30:377-391.

Marlida Y, Delfita R, Gusmanizar N, Ciptaan G (2010). Identification Characterization and Production of Phytase from Endophytic Fungi. World Acad. Sci. Eng. Technol. 65:1043-1046.

Meena P, Tripathi AD, Srivastava SK, Jha A (2013). Utilization of agro-industrial waste (wheat bran) for alkaline protease production by Pseudomonas aeruginosa in SSF using Taguchi (DOE) methodology. Biocatal. Agric. Biotechnol. 2:210-216.

Mittal A, Singh G, Goyal V, Anita Y, Aggarwal NK, Kumar N (2011). Optimization of medium components for phytase production on orange peel flour by *Klebsiella* sp. DB3 using response surface methodology. Inov. Romnian Food Biotechnol. 9:35-44.

Monteiro PS, Guimarães VM, de Melo RR, de Rezende ST (2015). Isolation of a thermostable acid phytase from *Aspergillus niger* UFV-1 with strong proteolysis resistance. Braz. J. Microbiol. 46:251-60.

Morales GA, Moyano FJ, Marquez L (2011). *In vitro* assessment of the

effects of phytate and phytase on nitrogen and phosphorus bioaccessibility within fish digestive tract. Anim. Feed Sci. Technol. 170:209-221.
Pal A, Khanum F (2010). Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. Bioresour. Technol. 101:7563-7569.
Pandey A, Szakacs G, Soccol CR, Rodriguez-León JA, Soccol VT (2001). Production, purification and properties of microbial phytases. Bioresour. Technol. 77:204-214.
Romero C, Salas M, García AC, Mendoza G, Plata F, Cervantes M, Viana T, Morales A (2009). Effect of phytase from *Aspergillus niger* on nutrient digestibility and activity of trypsin and chymotrypsin in weanling pigs. Arch. Zootec. 58:363-369.
Saad N, Esa NM, Ithnin H, Shafie NH (2011). Optimization of optimum condition for phytic acid extraction from rice bran. African J. Plant Sci. 5:168-175.
Shivanna GB, Venkateswaran G (2014). Phytase production by *Aspergillus niger* CFR 335 and *Aspergillus ficuum* SGA 01 through submerged and solid-state fermentation. Sci. World J. 2:1-6.
Singh B, Satyanarayana T (2011). Phytases from thermophilic molds: Their production, characteristics and multifarious applications. Process Biochem. 46:1391-1398.
Soni SK, Magdum A, Khire JM (2010). Purification and characterization of two distinct acidic phytases with broad pH stability from *Aspergillus niger* NCIM 563. World J. Microbiol. Biotechnol. 26:2009-2018.
te-Biesebeke R, Ruijter G, Rahardjo YSP, Hoogschagen MJ, Heerikhuisen M, Levin A, Van Driel KG, Schutyser MI, Dijksterhuis J, Zhu Y, Weber FJ, De Vos WM, Van Den Hondel KMJJ, Rinzema A, Punt PJ, Hondel KAMJJ, Van Den Rinzema A, Punt PJ (2002). *Aspergillus oryzae* in solid-state and submerged fermentations. Progress report on a multi-disciplinary project. FEMS Yeast Res. 2:245-248.
Vats P, Bhushan B, Banerjee UC (2009). Studies on the dephosphorylation of phytic acid in livestock feed using phytase from *Aspergillus niger* van Teighem. Bioresour. Technol. 100:287-291.
Velayudhan DE, Heo JM, Dersjant-Li Y, Owusu-Asiedu A, Nyachoti CM (2015). Efficacy of novel 6-phytase from *Buttiauxella* sp. on ileal and total tract nutrient digestibility in growing pigs fed a corn-soy based diet. Anim. Feed Sci. Technol. 210:217-224.
Zhang L, Wang YY, Zhang C, Wang YY, Zhu D, Wang C, Nagata S (2006). Supplementation effect of ectoine on thermostability of phytase. J. Biosci. Bioeng. 102:560-563.

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Full Length Research Paper

Dormancy breaking and germination of cat thyme *Teucrium marum* (Labiatae)

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Cat thyme is an important medicinal plant used for treating many diseases. With renewed interest as arthropod deterrent and repellents, there is immediate need to cultivate cat thyme on large scale in the laboratory. To improve cat thyme seed germination, obstacles involved in its seed dormancy must be investigated. To address such challenges, mechanical abrasion and chemical treatments were used to enhance the rate of seed germination. The application of mechanical abrasion with low speed grinding using walnut husk to improve the germination of cat thyme seeds was studied. In addition, to improve the seed germination percentage, the seeds were further treated with different concentrations of hydrogen peroxide (2.5, 5, 10 and 15%). The results show that the treatment of 5% hydrogen peroxide concentration promoted the seed germination rate. After the aforementioned different treatments, Baggy method was used and it achieved 50% germination rate within 30 days. In conclusion, the germination tests showed that cat thyme seed germination improved with combination of mechanical abrasion and 5% hydrogen peroxide treatments.

Key words: Cat thyme, seed germination percentage, hydrogen peroxide.

INTRODUCTION

Cat thyme (*Teucrium marum*), which is native to Spain and certain areas of southern Europe, is a medicinal plant that belongs to the mint family and is a close relative of Germander. This fragrant plant has been traditionally used by various herbalists and has thus been introduced in other regions and areas across the globe. In regions of southern Europe, cat thyme can grow anywhere from 3 to 4 ft tall; however, in areas such as England, the plant will rarely reach a foot in height. This plant can be grown in dry soil and have leaves covered with hairs, giving it shade to withstand hot arid summers. Although, it often dies in the wintertime, the plant can still

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be grown with proper care and conditions such as mats and various coverings. The leaves of cat thyme are oval and broader towards the base, and the flowers are pink and spiky on one side. The flowers are hermaphrodite and self-fertile.

Cat thyme has been found to have several astringent properties which facilitate its use as a stimulant for different medical conditions. The plant has foremost been used for homeopathic treatment, such as in treating people diagnosed of polyps. It has also been known to cure nerve diseases and its powdered leaves, either mixed in other ingredients or taken alone as snuff, has been recommended for conditions involving the head. This is mostly due to its compound, Assarabacca, and nowadays most lavender flowers are being substituted with cat thyme. Many herbalists have even used the plant to homeopathically treat small thread-worms in children.

This wide medicinal use of *T. marum* is due to its rich complex of chemical composition and presence of different monoterpenes and essential oils. Due to the presence of these chemical substances, it provides defense mechanisms for plants from the injuries of parasitic and/or pathogenic organisms (Eisner, 2000). Generally, some seeds including those of cat thyme have a physical or natural chemical inhibitor to germination (or dormancy), in these cases, the seed will only germinate in natural habitats when conditions are favorable. The inhibitor can be overcome by pre-treatment (such as boiling water treatment, abrasion, stratification, fire, smoke and leaching) of the seed before sowing. Once breaking of the seed dormancy is successful, the seed is subsequently activated by some hydrolytic enzymes, biosynthesis of RNA, DNA, protein, polysaccharides, and lipids which are essential for good growth of seedling. There are limited literatures regarding breaking of dormancy for effective seed germination of cat thyme (Delgado-Sánchez et al., 2011; Finch-Savage et al., 2006; Flores et al., 2008; Nadjafi et al., 2005; Willis et al., 2014).

Therefore, the purpose of this study was to determine the seed dormancy characteristics of cat thyme, breaking of the seed dormancy (Bradbeer, 2013) with some artificial treatments and further evaluation of seed germination using Baggy method.

MATERIALS AND METHODS

Cat thyme seeds were collected from senescent inflorescences from Beltsville Field (USDA-ARS, Beltsville, MD). Seeds were air dried and stored at room temperature until required for use. The experiments were carried out in the laboratory. The full grain seeds were selected to test germination capacity.

Walnut husk abrasion

First, 0.3% $KMnO_4$ solution was used to disinfect the seeds (200 seeds) for 15 min, and washed thoroughly with water and dried at room temperature. Then, the cleaned seeds (approximately 100) were mechanically abraded using walnut husk. The abrasion treatments lasted for 50 s with low (500 rpm) grinding using regular mechanical grinder. The abrasion process was repeated for two more times after 5 min interval. Walnut husk was removed and abraded seeds were scanned under the microscope (40×) for either mechanical damage or breaking (Figure 1). One hundred intact seeds were stored in the freezer prior to germination experiment.

Hydrogen peroxide treatment

Abraded seeds (5 × 15) were further treated with 0, 2.5, 5, 10 and 15% concentration of hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) for 72 h at room temperature (23 to 25°C). Simultaneously, seeds without abrasion (5 × 15) were also treated with hydrogen peroxide at four different concentrations.

Seed germination

Treated cat thyme seeds in ten variations, including control, were evaluated for seed germination in the climate controlled (23 to 25°C) small green house at Beltsville Agriculture Research Center. The seeds were planted in black plastic tray (6 × 20), using 36 cm^3 potting soil (Premier Pro-Mix HP-CC Mycorrhizae, Quakertown, PA) in each cell. Five seeds per replicate were planted in a row. Seeded tray was watered every 48 h to avoid complete dry soil. Germination percentage (%) was measured by the number of normal germinated seeds versus the total number of tested seeds. In addition, rate of seed germination and time intervals with total of 30 days was recorded. The data in triplicate samples was analyzed.

RESULTS AND DISCUSSION

In this investigation, the treatment of 5% hydrogen peroxide concentration promoted the seed germination rate (Table 1). After the above different treatments, baggy method was also used and achieved 50% germination rate within 30 days for 5% hydrogen peroxide treated abraded seeds. Since results were statistically inconclusive prior to the 30 days, no rate of germination was determined. The germination post 30 days of seeding exhibited results showing clear advantage of treatments combining walnut husk abrasion and treatment of hydrogen peroxide. All combined treatments exhibited more than 30% germination. The 5% hydrogen peroxide treatment of abraded seeds showed the best germination rate for hard to sprout cat thyme seeds in the green house condition.

Effect of walnut husk abrasion on seed germination

The seeds of cat thyme often face underdevelopment due to the presence of very hard seed coats, thick testa, pericarps and other structures that cause a high degree of mechanical resistance to the non-dormant embryo, and even block water and oxygen intake into the seeds.

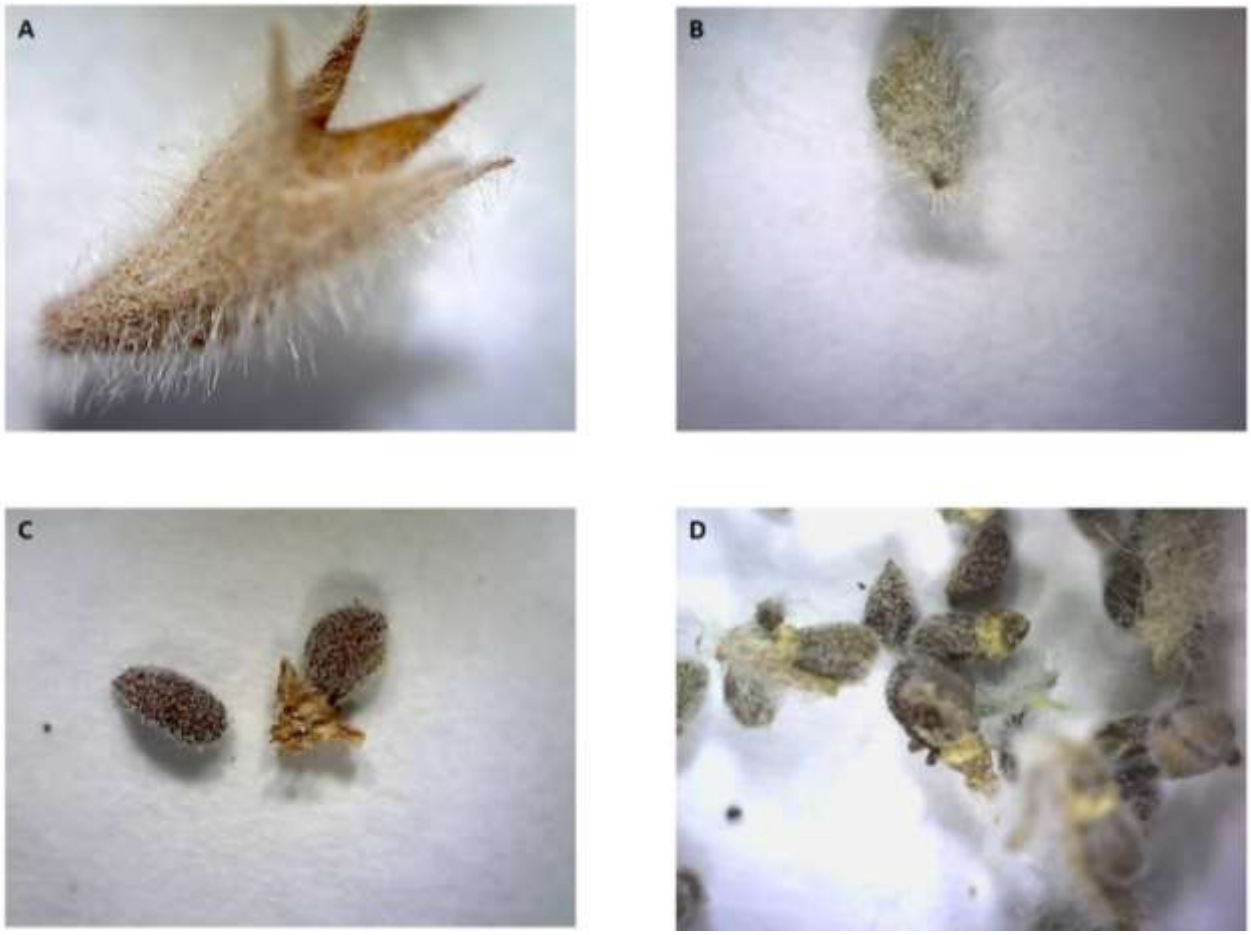

Figure 1. Preparation of cat thyme seed for germination. **A.** Cat thyme flower with seeds. **B.** Cat thyme seed separated. **C.** Cat thyme seeds stratified with walnut husk. **D.** Cat thyme seeds treated with hydrogen peroxide.

This poor germination due to physical and chemical characteristics has been seen in several research studies and has proven the lack of permeability of the seed to water, gases and solutes (Bradbeer, 2013; Agboola et al., 2006; Kozlowski, 1971). To overcome these problems, walnut husk abrasion was used to further enhance the seed coat permeability and seed germination rate. Hydrogen peroxide treatment was used on viable seeds to increase seed uptake of water and other constituents which in turn may induce more germination. The method of abrasion showed 30% more seed germination (Figure 1).

Ajiboye et al. (2009) investigated abrasion with emery cloth in the seed samples of four tree species which are: *Tamarindus indica* (L.), *Parkia biglobossa* (Jacq.), *Albizia lebbeck* (Benth) and *Prosopis africana*. The authors reported varied seed germination due to the treatment of emery cloth abrasion seed pretreatments. Several pretreatments were employed to soften, puncture and further split the hard seed coat in order to enhance the embryo for germination.

Agboola (1995) also used emery cloth abrasion method to enhance seed germination of *P. africana*. Several peer reviewed publications reported the abrasion method induction in several crop seeds. Ren and Tao (2004) investigated different methods including abrasion, sulphuric acid treatment, boiling water, cold stratification and seed exudate treatments for the germination of ten *Calligonum* species seeds. The authors reported that the rate of germination of seeds in response to different pretreatments was similar for all ten *Calligonum* species. However, the percentage of germination of seeds of the ten species was lowest for exudate treatments and highest for abrasion treatments. In addition, the abrasion, sulphuric acid and cold stratification treatments significantly enhanced overall germination rate. The treatment of cold stratification could break the dormancy of viable *Calligonum* seeds and thus initiate germination, but it has lethal effect on viable seeds like the boiling water treatments. Almost all germination parameters showed significant differences between the pre-sowing treatments for all ten *Calligonum* species.

Table 1. Seed germination observed after 30 days seeding.

Seed treatment	Replicate germination (no. 1)	Replicate germination (no. 2)	Replicate germination (no. 3)	Germination total of 15 seeds (%)
Control seeds	1	2	0	3 (20)
Abraded seeds	2	1	1	4 (23)
Control seed + 2.5% HP	1	1	2	4 (23)
Control seed + 5% HP	2	2	1	5 (33)
Control seed + 10% HP	1	2	0	3 (20)
Control seed + 15% HP	2	0	0	2 (13)
Abraded seeds + 2.5% HP	3	1	2	6 (40)
Abraded seeds + 5% HP	4	2	4	10 (66)
Abraded seeds + 10% HP	3	0	3	6 (40)
Abraded seeds + 15% HP	2	1	2	5 (33)

HP, hydrogen peroxide.

Shaik et al. (2008) investigated different treatments such as physical, mechanical and chemical pre-sowing in seeds of cancer bush (*Sutherlandia frutescens* L.) for germination response. The authors reported that soaking the intact seeds for 30 min in concentrated sulphuric acid (H_2SO_4) resulted in high germination percentage of 97.5% on the 14th day of culture. Similarly, seed dormancy was also completely broken by other method (mechanical scarification) in which 100% germination was obtained in day 2 of culture. The authors concluded that *S. frutescens* seeds possess exogenous dormancy due to the hard seed coat which is the main inhibitor of germination.

Clemens et al. (1977) studied the effect of a manual chipping treatment in addition to exposure of water at various temperatures for discrete time intervals on germination of seeds of five *Acacia* spp. The authors evaluated different parameters based on the percentage and rate of germination, and time duration for germination to commence. The authors found differences in response of the *Acacia* species to hot water treatments which was significant. Furthermore, increasing severity of treatments helped to improve germination rate and percentage germination up to a point where seed mortality became apparent. In addition, manual chipping of the seeds gave higher improvements in germination rate, and the seeds began to germinate faster than those given hot water treatment. However, in some species, germination percentages were lower in chipped seeds than in those treated with hot water.

Effect of hydrogen peroxide treatment on seed germination

Treatment of inorganic acids and peroxy acids in different concentration is another approach for efficient seed germination. Depending on the seed coat thickness and the type of the seed, different acids like sulfuric or nitric acids eliminate seed dormancy (Brandel, 2004; Baskin, 1998; Black and Bewley, 2000; Kapland, 1996). Exterior of the seed, including seed skin, shell and coating becomes porous when treated with acids, allowing penetration of water to dewatering process.

There are several successful reports on improvement of germination of variety of seeds using different concentrations of sulfuric acids (Aliero, 2004; Sxitus et al., 2003; Rehman et al., 1999; Rana and Nuatiyal, 1989; Uzen and Aydin, 2004; Saberi and Shahrian, 2011). For *Parkia biglobosa* seeds, the authors concluded that infusing seeds in sulfuric acid (98%) for 3 min as compared to other treatments showed highest level of germination percentage (50%), while sulfuric acid 90% treatment for 3 and 5 min led to, respectively 28 and 0% germination (Aliero, 2004).

On breaking dormancy of *Ulex europaeus* seeds, which have hard crust, the authors concluded that sulfuric acid and sand paper treatments caused an increase in germination of the seeds (Sxitus et al., 2003; Leubner-Metzger, 2003). Rehman et al. (1999) investigated the seed dormancy of *Acacia salicina* which have hard crust, and reported that sulfuric acid increased (98%) seed germination and increase in time of connection with this acid solution resulted in more number of buds. They concluded that seed treatment with sulfuric acid for 10 min showed 98% breaking seed dormancy but seed infusion for 30 min in the mentioned acids showed significant germination increase.

Rana and Nuatiyal (1989) also conducted similar experiment on the seeds of *Acacia farniesiana*; they observed that using sulfuric acid caused increase in germination, but with increase in time of seed connection with acid, there was injury to embryo structure, therefore producing more abnormal seedling. Uzen and Aydin

(2004) reported that scratching of seeds showed significantly positive effect on breaking dormancy and germination stimulus of seeds of *Medicago* genus. Saberi and Shahrian (2011) investigated the effect of different treatments on breaking seed dormancy, germination stimulus and growth of *Citrullus colocynthis* seeds. The treatments included sulfuric acid (98%) in 20 and 40 min intervals, potassium nitrate (0.2%) within 72 h, hot water at 90°C during 10 min and scratching by sand paper. To compare these treatments and normal germination, distilled water was used as control. The experiments were performed using a completely randomized design with four repetitions and six treatments. Results of variance analysis and mean comparison showed that there were significant statistical differences (0.01 levels) between treatments for percentage and velocity of germination, length of the root and shoot. The maximum percentage and velocity of germination and length of the shoot obtained in scratching by sand paper treatment and results of other treatments are in lower level. According to the results obtained, scratching is the most suitable method for dominance of seed dormancy of *C. colocynthis* species. The use of acid/alcohol treatments could be termed the chemical method of scarification to enhance germination in savanna tree seeds. This method was found to improve germination in both tropical and savanna tree seeds.

Conclusion

Different treatments for enhancement of cat thyme seed germination were evaluated. The results suggest that germination of cat thyme seed improved with combination of walnut husk induced mechanical abrasion and 5% hydrogen peroxide treatments. This methodology could potentially be useful for other crop seeds with physical or chemical inhibitor for germination.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Agboola DA (1995). Studies on dormancy and germination of seeds of *Prosopis africana*. Nig. J. Bot. 8:45-56.

Agboola DA, Ebofin AO, Aduradola AM, Ajiboye AA (2006). Effect of pre-sowing treatments on seed germination of two savanna tree legumes. J. Trop. For. 20:1-11.

Ajiboye AA, Atayese MO, Agboola DA (2009). Effect of presowing treatments on seed germination and percentage starch content levels in *Tamarindus indica, prosopis africana, parkia biglobossa* and *Albizia lebbeck* . J. Appl. Sci. Res. 5(10):1515-1519.

Aliero BL (2004). Effects of sulphuric acid, mechanical scarification and wet heat treatments on germination of seeds of *Parkia biolobosa*. Afr. J. Biotechnol. 3:179-181.

Baskin CC, Baskin JM (1998). Seeds, Ecology and Evolution of Dormancy and Germination. Academic Press, New York. Vol. l6, pp.101-106.

Black M, Bewley JD (2000). Seed Technology and its Biological Basis, CRC Press, 2000 NW Corporate Blvd., Boca Raton, FL 33431-9868. Hardback. 419p.

Bradbeer JW (2013). Seed Dormancy and Germination, Springer Science & Business Media, 146p.

Brandel M (2004). The role of temperature in the regulation of dormancy and germination of two related summer-annual mudflat species. Aquat. Bot. 79:15-32.

Clemens J, Jones PG, Gilbert NH (1977). Effect of seed treatments on germination in *Acacia*. Aust. J. Bot. 25:269-276.

Delgado-Sánchez P, Ortega-Amaro MA, Jiménez-Bremont JF and Flores J (2011). Are fungi important for breaking seed dormancy in desert species? Experimental evidence in *Opuntia streptacantha* (Cactaceae). Plant Biol. 13:154-159.

Eisner T, Eisner M, Aneshansley DJ, Wu C-Li, Meinwald J (2000) Chemical defense of the mint plant, *Teucrium marum (Labiatae)*. Chemoecology 10:211-216.

Finch-Savage WE and Leubner-Metzger G (2006). Seed dormancy and the control of germination. New Phytol. 171:501-523.

Flores J, Jurado E. and Jiménez-bremont JF (2008). Breaking seed dormancy in specially protected *Turbinicarpus lophophoroides* and *Turbinicarpus pseudopectinatus* (Cactaceae). Plant Species Biol. 23:43-46.

Kapland LO (1996). Principles of seed science and technology. Translated by Sarmadnia Gh. Jahaddaneshgahi, Mashhad publications. 288p.

Kozlowski TT (1971). Growth and development of trees. Academic Press Ltd. London, 1:71-81.

Leubner-Metzger G (2003). Functions and regulation of β-1,3-glucanase during seed germination, dormancy release and after-ripening. Seed Sci. Res. 13:17-34.

Nadjafi F, Bannayan M, Tabrizi L, Rastgoo M (2005). Seed germination and dormancy breaking techniques for Ferula gummosa and *Teucrium polium* J. Arid Environ. 64(3):542-547.

Rana U, Nuatiyal AR (1989). Seed dormancy in *Acacia farnesiana*, Seed Research. 17:122-127.

Rehman S, Loescher RN, Harris PJC (1999). Dormancy breaking and germination of *Acacia saliciina* seeds. Seed sci. Technol. 27:553-57.

Ren J, Tao L (2004). Effects of different pre-sowing seed treatments on germination of 10 Calligonum species. Forest Ecol. Manage. 195(3):291-300.

Saberi M, Shahriari A, Tarnian F, Noori S (2011). Comparison the Effect of Different Treatments for Breaking Seed Dormancy of *Citrullus colocynthis.* J. Agric. Sci. 3(4):61-67.

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

Sxitus CR, Hill GD, Scoot RR (2003). The effect of temperature and scarification method on *Ulex europaeus* seed germination. New Zealand Plant Protect. 56:201-205.

Uzen F, Aydin I (2004). Improving germination rate of Medicago and Terifolium species. Asian J. Plant Sci. 3:714-717.

Willis CG, Baskin CC, Baskin JM, Auld JR, Venable DL, Cavender-Bares J, Donohue K, Rubio de Casas R and The NESCent Germination Working Group (2014). The evolution of seed dormancy: environmental cues, evolutionary hubs, and diversification of the seed plants. New Phytol. 203:300-309.

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