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African Journal of Biotechnology

Full Length Research Paper

Synthesis of silver nanoparticles using crude leaf extracts of *Acacia nilotica, Azadirachta indica*, *Carissa spinarum, Melia azedarach, Senna didymobotrya* and *Warburgia ugandensis,* and their antifungal activity against *Sporisorium scitamineum*

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Bio-synthesised silver nanoparticles are effective in controlling several micro-organisms. They are correspondingly environmentally friendly, affordable, and easy to synthesise when compared with chemically synthesised silver nanoparticles. This study investigated the efficacy of biosynthesized silver nanoparticles against the fungus *Sporisorium scitamineum*, the causal agent of sugarcane smut. The reduction of silver nitrate upon mixing with the plants' crude extracts was evidenced by the change in colour of the mixture to dark brown. Optimization of the mixtures using ultraviolet-visual spectroscopy showed peaks in the range of 340 to 450 nm. The Fourier transform infrared spectroscopy analysis identified proteins to be essential capping agents, and reducing sugars were responsible for the reduction of silver nitrate to nanoparticles and stabilizing the nanoparticles. The transmission electron microscope analysis showed the sizes of the nanoparticles to vary between 3 and 70 nm. *Carissa spinarum* and *Melia azedarach* had the most antifungal activity against *S. scitamineum* as observed from the inhibition-zone assay. Silver nanoparticles were successfully synthesized using the selected botanicals. All the synthesized nanoparticles showed varying antifungal effects against the *S. scitamineum. C. spinarum* and *M. azedarach* exhibited the highest antifungal activity, while *Azadirachta indica* showed the least.

Key words: *Sporisorium scitamineum*, *Acacia nilotica, Carissa spinarum, Senna didymobotrya, Warburgia ugandensis, Melia azedarach, Azadirachta indica,* bio-synthesised silver nanoparticles, antifungal activity.

INTRODUCTION

Traditionally, silver has been known and used for its antimicrobial activity (Jamiu and Bello, 2018). When reduced to their nano-form, silver nanoparticles (AgNPs) possess novel and more efficient antimicrobial properties, owing to their large surface to volume ratio, size, shape and structure (Rafique et al., 2017).

Metal nanoparticles have been traditionally produced by physio-chemical methods that include ion sputtering or pulsed laser ablation, reduction, solvothermal synthesis, hydrothermal and sol-gel methods. Recently, there have been environmentally friendly synthesis methods that use natural products that have been termed the “green synthesis” or “biosynthesis” of nanoparticles (Chouhan, 2018; Vala et al., 2021).

Chemical and physical methods are generally expensive, harmful and inflammable, unlike the biosynthesis method which is cost-effective, energy-saving and environmentally benign as it uses microorganisms and plant extracts. The phytochemicals such as lipids, proteins, polyphenols, carboxylic acids, saponins, amino acids, polysaccharides, and enzymes present in biological material are used as reducing, capping and stabilizing agents. The use of agricultural waste helps in reducing the cost of producing the AgNPs as well as limiting the need of using hazardous chemicals and therefore encourages the “green synthesis” production (Chouhan, 2018; Hemlata et al., 2020). The use of plant extracts has been proven to be affordable, easy to bulk up, simple and environmentally friendly (Sanchooli et al., 2018; Raza et al., 2021).

Biosynthesised silver nanoparticles (b-AgNPs) have been found to have antibacterial, antifungal, and antiviral properties, with no environmental concerns and development of microbial resistance. These characteristics have ignited increasing interest in the synthesis of silver nanoparticles (Velu et al., 2017; Hemlata et al., 2020).

Upon synthesis of the AgNPs are synthesised, it is essential to characterise them to understand their physicochemical properties which could have an impact on their biocompatibility during use. The characterization is aimed at understanding the size, size distribution, shape, surface area, stability and aggregation of the particles (Zhang et al., 2016). Characterizing can be done through various analytical techniques which include ultraviolet-visible (UV-Vis) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, X-ray diffractometry (XRD), dynamic light scattering (DLS), transmission electron microscopy (TEM) and atomic force microscopy (AFM) (Khatoon et al., 2017; Al-zubaidi et al., 2019; Jamiu and Bello, 2018; Khan and Javed, 2021).

Upon characterising the b-AgNPs, they can then be used for various purposes which include using them as an antifungal treatment. Overcoming fungal diseases is difficult, especially because of the limitation of several antifungal remedies as well as the environmental impacts that are caused by chemical treatments (Zhang et al., 2016).

Biosynthesised silver nanoparticles have shown exceptional antifungal activity against several phytopathogenic fungi including *Rhizoctonia solani, Alternaria alternata, Sclerotinia sclerotiorum, Botrytis cinerea, Macrophomina phaseolina* and *Curvularia lunata* at the concentration of 15 mg (Zhang et al., 2016).

Several plants have been found to possess medicinal and antimicrobial properties. These properties are attributed to the presence of phytochemicals such as limonoids, flavonol glycosides, saponins, steroids, terpenes, and tannins (Ibrahim et al., 2010; Jafari et al., 2013; Okello and Kang, 2019). The leaf extracts may possess variable effects on pathogens, but have been found to have a higher efficacy when they are used as b-AgNPs (Chouhan, 2018).

The objective of this study is to develop, characterise, and evaluate AgNPs synthesised from the leaf extracts of known antimicrobial plants against *Sporisorium scitamineum* (Syd) M. Piepenbr., M. Stoll & Oberw., the fungal pathogen that causes sugarcane smut (Bhuiyan et al., 2021). Hitherto, only *Azadirachta indica* and *Melia azedarach*, among the selected botanicals, have been used to synthesize AgNPs, yet their antifungal efficacy has not been evaluated against *S. scitamineum.*

MATERIALS AND METHODS

Sourcing plant extracts

This study was done in Kenya and Eswatini from 2020 to 2021. The selection of the plants to be used was influenced by their known medicinal properties as well as availability (Abdel-Rahim et al., 2016; Berhanu and Babele, 2020; Carpinel and Alonso, 1999; Hasan et al., 2019; Jeruto et al., 2016; Okello and Kang, 2019). The selected plants were *Warburgia ugandensis, Carissa spinarum, Acacia nilotica, A. indica*, *M. azedarach* and *Senna didymobotrya*. The *M. azedarach* extracts were sourced from Eswatini and the other five plant leaves were sourced from the Jomo Kenyatta University of Agriculture and Technology’s (JKUAT) botanical garden, in Kenya.

Plant extract preparation

The plant crude extract preparation was done by cleaning the leaves with sterile water, drying them and cutting them into small pieces using a blender. Then 50 g of each leaf sample was heated at 80°C in 250 ml of sterile water in a 500 ml Erlenmeyer flask for 30 min. The crude leaf extracts were then filtered using Whatman No. 1 and stored at 4°C (Velu et al., 2017).

Biosynthesis of nanoparticles

To synthesize the AgNPs, 1 mM of silver nitrate was formulated by adding 0.167 g of silver nitrate into 1 L of distilled water. The mixture of the silver nitrate and the plant’s crude extract was made

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at an optimised ratio and kept in darkness, to prevent photo-reduction of the silver, at 28^{o}C in a 150rpm shaking incubator. The intensity of the colour which is indicative of nanoparticle formation was recorded between 200 and 800 nm on a UV-Vis spectrophotometer using the Flight Deck, Jenway Model 6800 Spectrophotometer (Velu et al., 2017).

Optimizing the nanoparticles

The b-AgNPs were optimised under different reaction conditions which included leaf extract reaction volume (2, 3, 4, 5, 6, 7, 8, and 9 ml) and the duration of incubation of the AgNPs in darkness which was varied at 0, 2, 4, 12, 24, 48 and 72 h (Houllou et al., 2019). While optimizing each parameter, the other parameters were kept constant.

The b-AgNPs were isolated from the optimized mixture by centrifugation at 12 000 rpm for 20 min. The pellet was then purified using distilled water and washed twice to ensure better separation of free entities from the AgNPs. The b-AgNPs were kept at -20°C for 24 h, moved to -80°C to be kept for 48 h, and then they were lyophilized and used for further characterization (Velu et al., 2017).

Characterizing the nanoparticles

UV-Vis Spectra analysis

A sample (1 ml) of the suspension was collected periodically to monitor the completion of bio-reduction of Ag+ in an aqueous solution. The UV-Vis spectrum of the solution was measured between wavelengths 200 and 800 nm using the Jenway Model 6800 Spectrophotometer Flight Deck with a resolution of 1 nm (Sanchooli et al., 2018).

FTIR analysis

The nanoparticle characterization included ascertaining the active biomolecules responsible for the reduction; capping and stabilising by Fourier transform infrared (FTIR) spectrometer model 8400, Shimadzu. For the FTIR analysis, the dried b-AgNPs were added to FTIR-grade potassium bromide (KBr) in 1: 100 ratios and observed in the range of 4000 to 400 cm^{-1} (Mondal et al., 2020).

TEM analysis

Analysis to determine the morphology, size and shape of the nanoparticles was done using the JEM-2100 Electron Microscopy. The TEM sample grid with a continuous silicon oxide film was prepared. The sample grid was then derivatized by exposing the silicon oxide to 10 µl of aminopropyldimethylethoxysilane solution. The b-AgNPs were then citrate-stabilized for them to have a negative charge to be attracted to the positively charged TEM surface grid (Bonevich and Haller, 2010).

Collection and identification of the fungus

The smut-infected plants were identified at the Eswatini Sugar Association's experimental plots at Nsoko. Visible sori were cut from the infected sugarcane plants and bagged to prevent any spread to healthy plants. These spores were rinsed three times with distilled water and cultured in potato dextrose agar (PDA). The plates were incubated for fourteen days in darkness at 28°C (Cui et al., 2020; Singh et al., 2005). To purify the cultures, single colonies were transferred onto new plates and incubated in darkness at 28°C (Que et al., 2014).

Fungal genomic DNA was extracted using a Zymo Fungal/Bacterial Genomic DNA Extraction Kit (Inqaba Biotech, South Africa) following the manufacturer's instructions. The quality and concentration of DNA were analysed by 1% agarose gel electrophoresis and by using a nanodrop spectrophotometer.

To verify the identity of the fungus, the extracted DNA was amplified in conventional PCR using the *bE*4 (5'-CGCTCTGGTTCATCAACG - 3') and *bE*8 (5'-TGCTGTCGATGGAAGGTGT - 3') primers that are specific for *S. scitamineum* (Zhang et al., 2015).

Conventional PCR amplification was carried out in a 25 μL volume containing 1 μL DNA, 12.5 μL of 2x OneTaq master mix, 0.5 μL of each of the upstream and downstream primers and 10.5 μL of water. The PCR amplification was performed following a thermal cycling programme of 95°C for 5 min; 35 cycles of 95°C for 30 s, 50°C for 30 s, and 68°C for 40 s; and a final extension at 72°C for 5 min.

The PCR amplicons were checked for quality on a 1% agarose gel electrophoresis and then documented. A negative control sample only contained the master mix with no DNA template.

Screening of b-AgNP for antifungal activity

The silver nanoparticles that were produced from the crude extracts from the different plants were evaluated to select the b-AgNPs that had the highest antifungal activity against *S. scitamineum*. Fungal spores suspended in sterile water were spread onto PDA media using a sterile swab and then incubated for 24 h at 28°C. The disc method was used, with the standard antifungal nystatin (100 µg) as a positive control and distilled water as a negative control (Alyousef et al., 2019; Al-zubaidi et al., 2019; Hameed et al., 2015; Khan and Javed, 2021; Medda et al., 2015). The synthesised b-AgNPs were dissolved in distilled water and each b-AgNP treatment was evaluated at 2.5, 5 and 10 mg/ml in three replicates.

Data analysis

The antifungal efficacy of the various b-AgNPs was evaluated by measuring the zones of inhibition. Data were subjected to ANOVA and means were separated at P = 0.05.

RESULTS

Biosynthesis of silver nanoparticles

The reduction of the silver nitrate, upon mixing it with the crude extract, was seen by the colour change from light pale into dark brown (Figure 1).

Characterization of the b-AgNPs

UV-Vis spectroscopy

The analysis showed absorption peaks for the b-AgNPs that were made from the different botanicals at a range between 340 and 450 nm. To optimise the amount of crude leaf extract that was added to the 1 mM silver

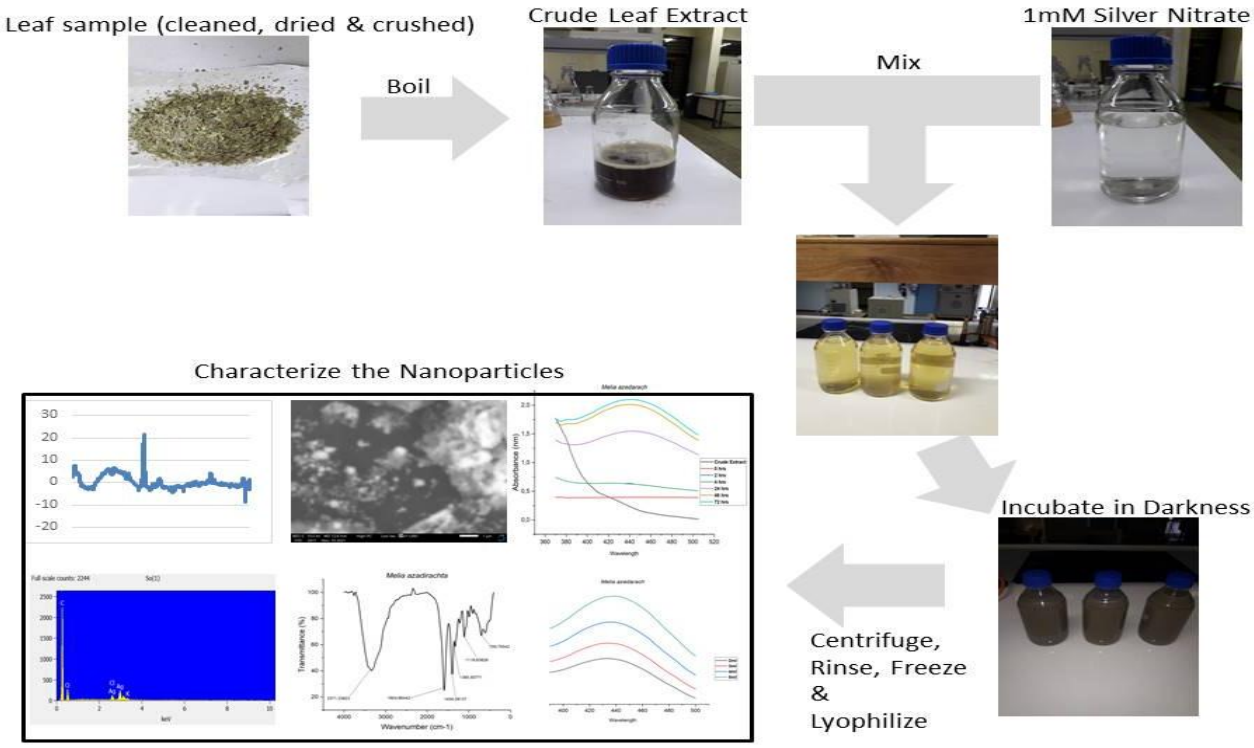

Figure 1. The biosynthesis of silver nanoparticles: by mixing the crude leaf extract with silver nitrate, incubating the mixture, centrifuge and lyophilise the AgNPs and then characterise the produced b-AgNPs
Source: Authors

nitrate ($AgNO^3$) to reduce it to AgNPs; 5 ml was added for *A. indica* and *Melia azedarach*, while 4 ml was added for *A. nilotica* and *S. didymobotrya,* 3 ml was added for *C. spinarum* and *W. ugandensis* and the plasmon peaks were observed at 430, 435, 385, 385, 335 and 385 nm, respectively (Figure 2). When optimizing for the required incubation period of the mixture; *A. indica, A. nilotica, M. azedarach* and *W. ugandensis* required to be incubated for 72 h, while *S. didymobotrya* required to be incubated for 48 h and *C. spinarum* required 24 h, and the plasmon peaks were observed at 390, 385, 440, 390, 395, and 390, respectively (Figure 3).

Fourier transform infrared (FTIR) analysis

The FTIR analysis of the b-AgNPs (Figure 4) shows the bands that correspond with the biomolecules responsible for the reduction of $AgNO_3$ to nanoparticles. The bands were observed at 3355 to 3402 cm^{-1}, 1595 to 1605 cm^{-1}, 1404 cm^{-1}, 1200 to 1300 cm^{-1}, 995 to 1118 cm^{-1}, and at 500 to 720 cm^{-1}.

TEM analysis

The sizes of the b-AgNPs that were synthesized using the various botanicals varied between 3 and 70 nm. *C. spinarum* produced AgNPs with the size range of 3 to 33 nm, *M. azedarach* produced 9 to 70 nm, while *A. indica, A. nilotica, S. didymobotrya* and *W. ugandensis* produced 14 to 53 nm, 14 to 52 nm, 6 to 35 nm and 12 to 53 nm, respectively (Figure 5 and Table 1). The shape and surface texture of the b-AgNPs was consistently spherical and smooth for all the nanoparticles (Table 1).

Verification of the fungi

To verify the identity of the fungus, the collected samples were screened by conventional PCR using the *S. scitamineum* specific primers *bE*4 and *bE*8. The samples produced an amplicon of 459 bp which corroborated the results by Izadi and Moosawi-jorf (2007).

Screening of nanoparticles for antifungal activity

The evaluation of the antifungal activity of the different b-AgNPs showed that *C. spinarum* and *M. azedarach* had higher inhibition zones, but *C. spinarum* was shown to be superior at both 5 and 10 mg/ml (Figure 6). *A. indica* recorded the least antifungal activity at all the concentrations, while the other b-AgNPs had a moderate

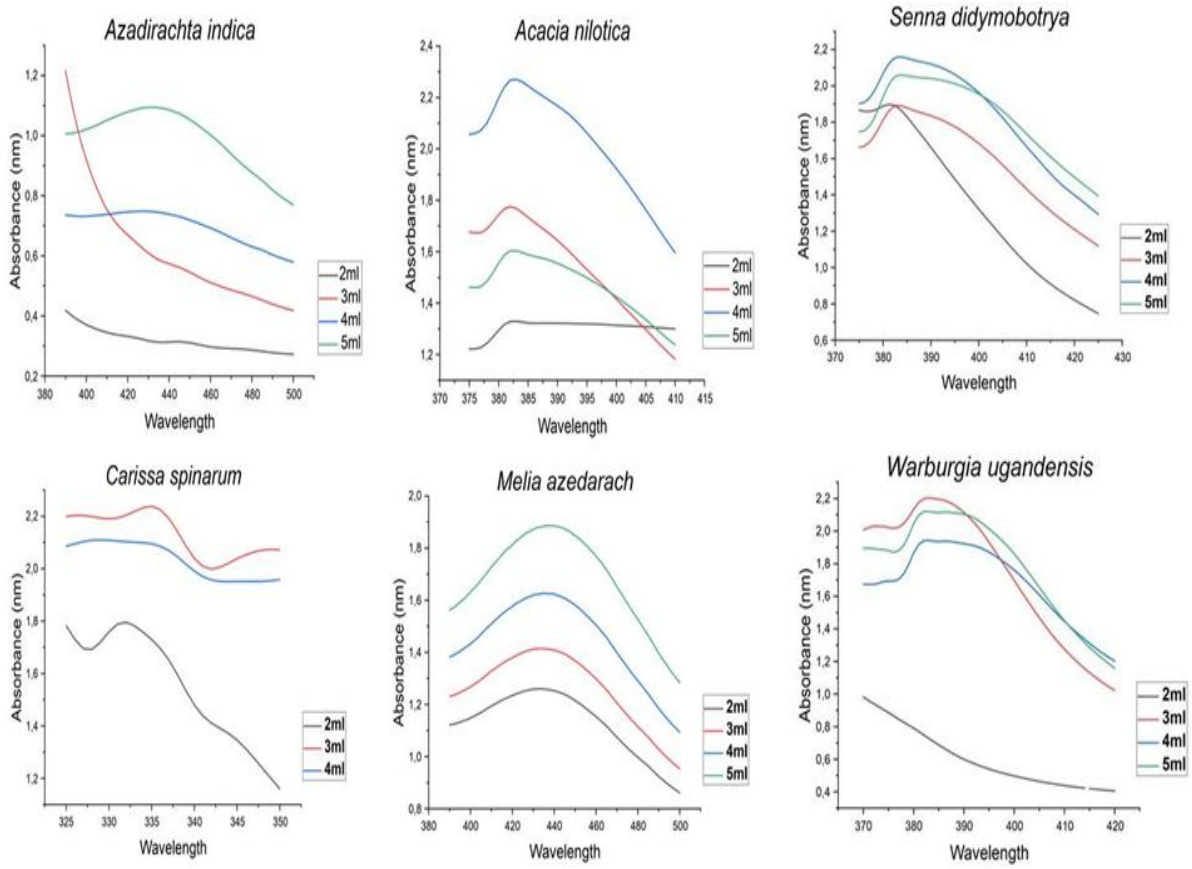

Figure 2. The optimization of the b-AgNPs by varying crude extract amounts of the six plants, observing the absorbance at a wavelength from 200 to 800 using a UV-Vis spectroscopy.
Source: Authors

effect.

DISCUSSION

The colour change upon mixing the silver nitrate with the crude extract is indicative of the formation of the nanoparticles by the reduction of the $AgNO_3$ by the crude leaf extract to form b-AgNPs (Alyousef et al., 2019; Sharma et al., 2014; Velu et al., 2017). The colour change is due to the occurrence of the Surface Plasmon Resonance (SPR) phenomenon which is caused by the interaction of the conduction electrons of the silver nanoparticles (Sharma et al., 2014; Vala et al., 2021). The phytochemicals (lipids, proteins, polyphenols, carboxylic acids, saponins, amino acids, polysaccharides and enzymes) present in plants are used as reducing, capping and stabilising agents (Chouhan, 2018).

During the synthesis of the b-AgNPs, the amount of crude extract that was added as well as the incubation period were optimised by UV-Vis spectroscopy. The optimization process produced absorption peaks for the b-AgNPs at a range between 340 and 450 nm and confirms the formation of the nanoparticles, which is consistent with the findings of Alyousef et al. (2019), Sanchooli et al. (2018) and Masum et al. (2019). Namratha and Monica (2013) reported the range of the peaks that indicate the formation of nanoparticles to be observed between 350 and 550 nm. The various botanicals required varying amounts of the leaf extract as well as incubation periods to achieve the optimum formation of the b-AgNPs. This variation could be caused by the changeable levels of phytochemicals that are contained in the different botanicals. This variation of phytochemicals could have resulted in a variable reduction rate of the silver nitrate as well as the variable capping and stabilizing of the nanoparticles (Chouhan, 2018).

The FTIR analysis of b-AGNPs validates the activity of biomolecules that are in charge of the reduction and stabilization of the b-AgNPs (Khatoon et al., 2017; Mondal et al., 2020). The analysis shows the bands between 3355 and 3402 cm^{-1} which correspond to N-H stretching of the proteins' secondary amide. The peaks at 1596-1605 cm^{-1} indicate stretch vibrations for the –C=C- bond, whilst the Benzene ring C=C and C-C are shown

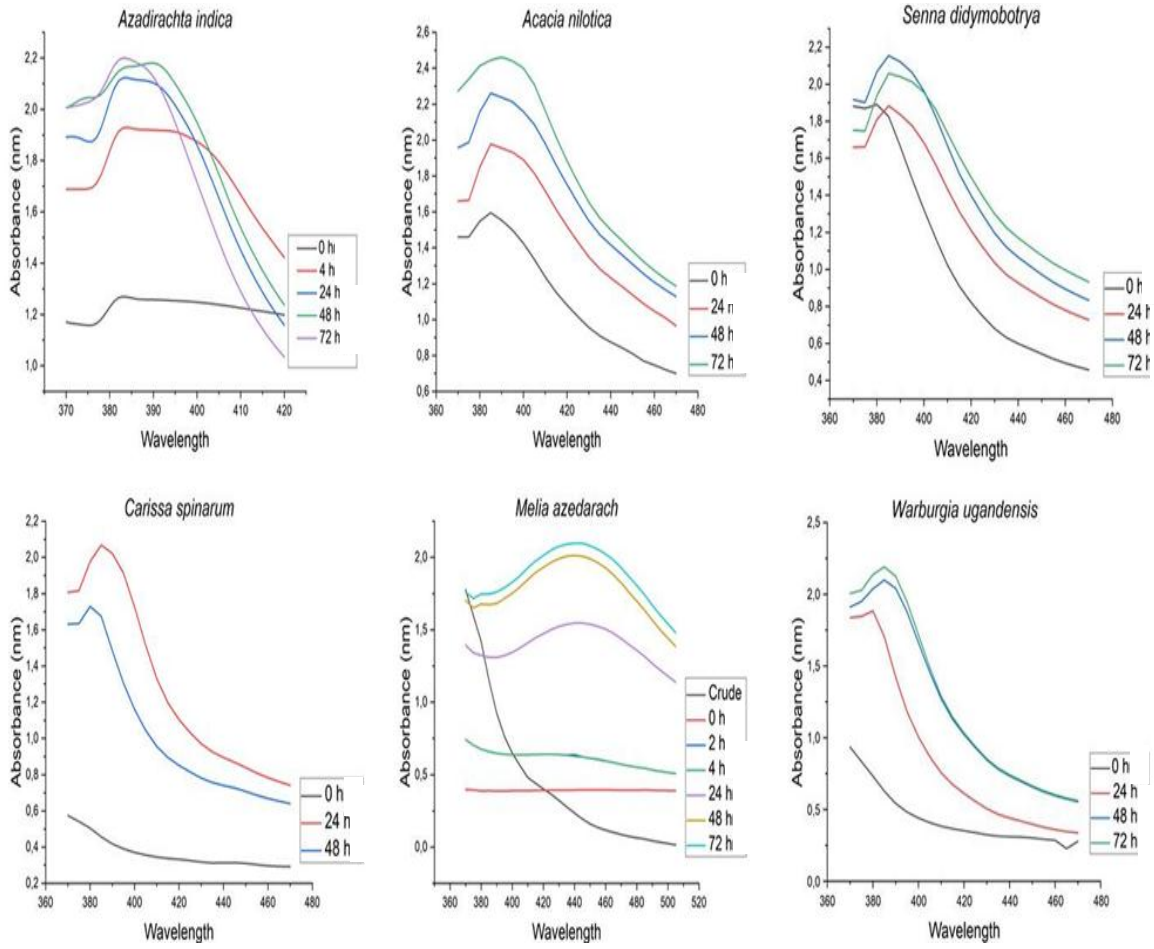

Figure 3. The optimization of the b-AgNPs by varying the incubation period, observing the absorbance at a wavelength from 200 to 800 using a UV-Vis spectroscopy.
Source: Authors

by peaks at 1404 cm^{-1}. The C-H bond in the pyridine ring appears at 1200-1300 cm^{-1} and the C-OH phenols appear at 995-1118 cm^{-1}. The peaks at 500-720 cm^{-1} show the presence of the AgNPs (Al-zubaidi et al., 2019). The synthesised nanoparticles were surrounded by proteins and other functional groups such as terpenoids. These results indicate the strength of the carbonyl groups from the proteins and amino acids to bind with metal, thereby capping the AgNPs. The presence of the reducing sugars could indicate their responsibility in reducing the $AgNO_3$ to AgNPs and stabilizing the AgNPs (Khatoon et al., 2017).

The TEM analysis was able to determine the sizes, shapes and texture of the b-AgNPs. Nanoparticles, by their definition, should range between 1 and 100 nm (Vala et al., 2021; Mondal et al., 2020). Their nano-scale size, morphological substructure and shape are of great importance as they give the AgNPs the physicochemical properties that suit them for their multiple applications (Chouhan, 2018; Khatoon et al., 2017). The size and shape of the *A. indica* AgNPs were shown to be consistent with the sizes that are documented by Firdhouse and Lalitha (2015), Khatoon et al. (2017) and Namratha and Monica (2013) (Table 1). The synthesis and characterisation of AgNPs made from other botanicals had not been documented before this study.

The fungal isolate was positively verified by conventional PCR using the *S. scitamineum* specific primers *bE*4 and *bE*8 (Zhang et al., 2015). All the b-AgNPs had an inhibitory effect on the growth of the fungus *S. scitamineum*, but with varying efficacies which could be due to the difference in phytochemicals that reduce the silver nitrate to nanoparticles among the botanicals (Hussain et al., 2019). This was observed by the formation of inhibition zones in all the b-AgNP treatments. The positive inhibition of the growth of the fungus *in-vitro* warrants further *in-vivo* studies.

Conclusion

In this study, biosynthesized silver nanoparticles (b-

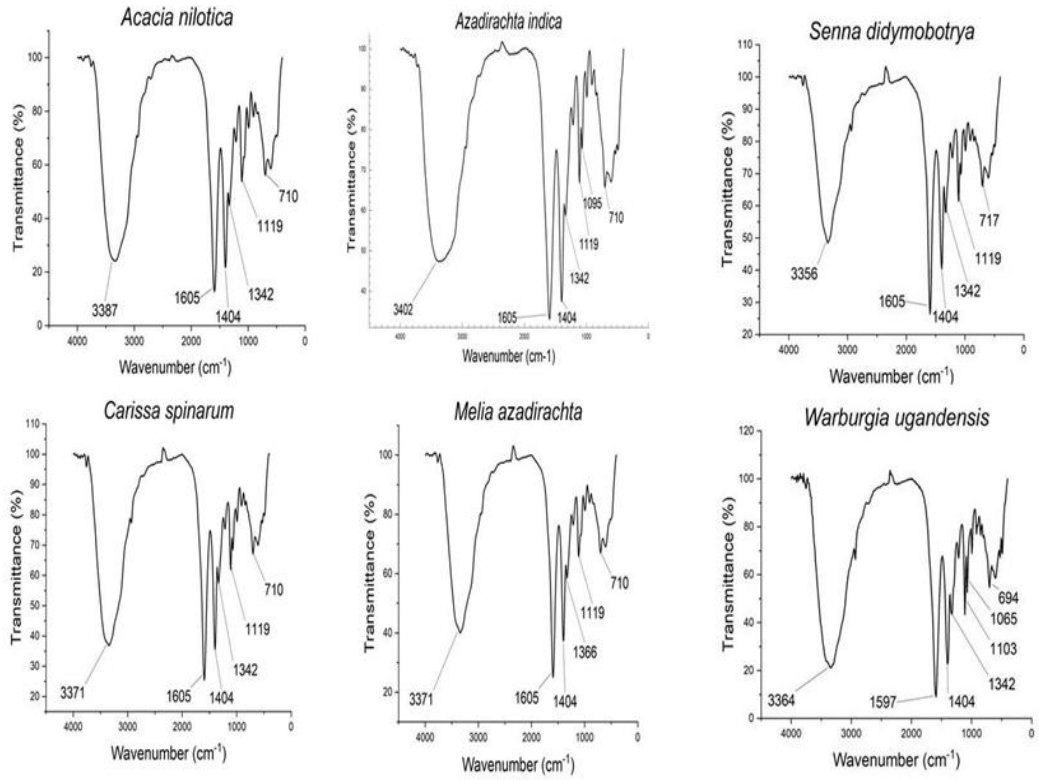

Figure 4. The FTIR results indicating the presence and sites (bands) of the biomolecules that are responsible for reducing the $AgNO^3$ to b-AgNPs as well as those responsible for capping and stabilising the AgNPs
Source: Authors

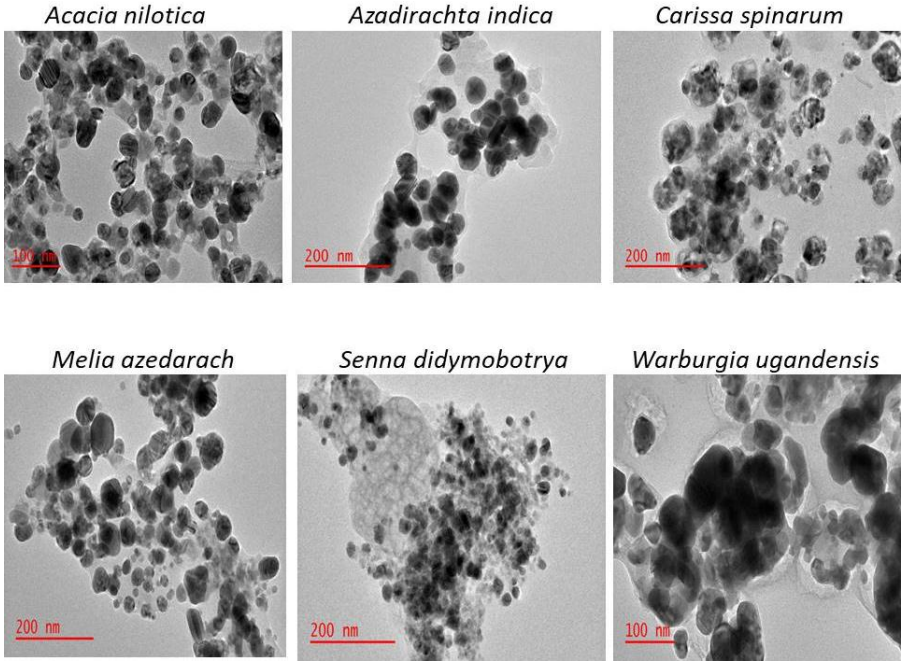

Figure 5. The TEM results indicating the sizes and shapes of the b-AgNPs that were produced using crude extracts from the different medicinal plants.
Source: Authors

Table 1. The size ranges, shapes and surface textures of the b-AgNPs that were produced using the six medicinal plants as observed by the TEM.

b-AgNPs	Size range (nm)	Shape	Surface texture
Acacia nilotica	14 - 52	Spherical	Smooth
Azadirachta indica	14 - 53	Spherical	Smooth
Carissa spinarum	3 - 33	Spherical	Smooth
Melia azedarach	9 - 70	Spherical	Smooth
Senna didymobotrya	6 - 35	Spherical	Smooth
Warburgia ugandensis	12 - 53	Spherical	Smooth

Source: Authors

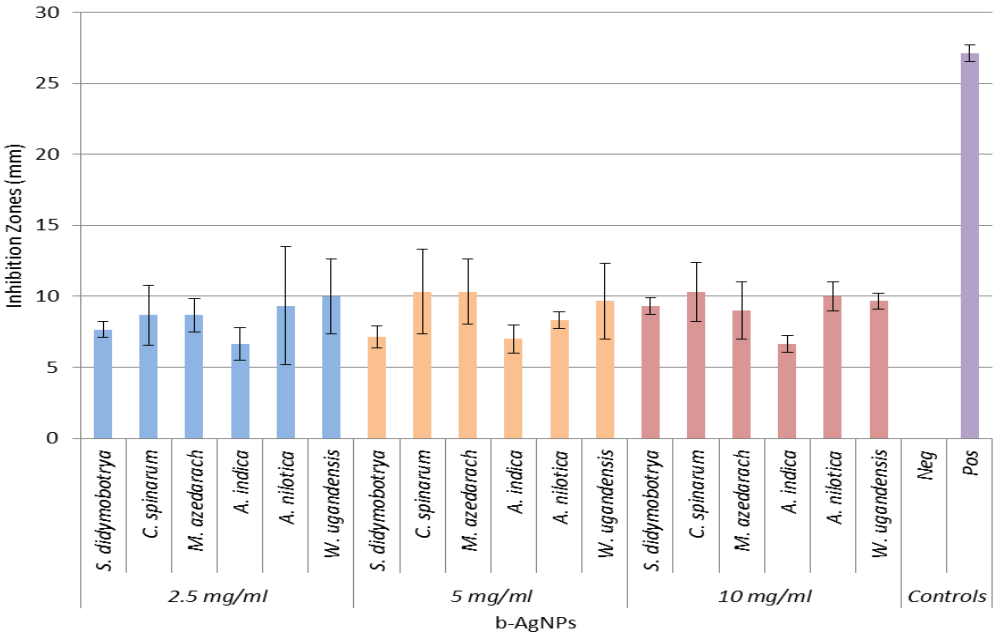

Figure 6. The antifungal effect of the b-AgNPs measured by their inhibition zones when tested in-vitro on *Sporisorium scitamineum* at 2.5, 5 and 10 mg/ml with nystatin as a positive control and distilled water as a negative control
Source: Authors

AgNPs) of varying sizes were successfully synthesized using *A. nilotica, C. spinarum, S. didymobotrya, W. ugandensis, M. azedarach* and *A. indica.* The synthesized nanoparticles were all spherical in shape, smooth in texture, and had a size range between 3 and 70 nm as indicated by the TEM, FTIR and UV-Vis characterization. All the biosynthesized nanoparticles showed antifungal effects against the fungus *S. scitamineum in-vitro. C. spinarum* and *M. azedarach* exhibited the highest antifungal activity, while *A. indica* showed the least.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Fatty acid profiles in Chinese Maiwa yak milk across the lactation cycle

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An experiment was conducted to determine fatty acid (FA) profiles in Chinese Maiwa yak milk across the lactation cycle. Sixty-one healthy, similar weight, 4-7 years old, 3-6 parity Maiwa yaks with unrelated background were selected randomly from Hongyuan County, Sichuan province, in southwest of China (with the altitude over 3500 m). The yaks graze on natural pasture all year around without irrigation, fertilizer, or other changes to the pasture. The samples for each animal were collected separately at 1, 2, 3, 4, 5, 6, 7, 15, 30, 60, 120 and 180 days postpartum for fatty acid composition analysis. The most abundant FA species in yak milk were C16:0, C18:1c9, C18:0 and C14:0, all of which varied significantly with the lactation periods. C10:0, C12:0, C14:0 and C16:0 was positively correlated with each other, and negatively correlated with C18:0. C18:2t9c11 was negatively correlated with saturated FA (SFA). C18:1t11, iso-C15:0, C22:0 and C16:1c7 were located in the central positions of the correlation network. C18:1t11 showed close correlation with C18 unsaturated fatty acid (UFA) (C18:1t13 and C18:3n3), C16:0, C19:0, and anteiso-C17:0. Besides with each other, iso-C15:0 and C22:0 were associated with C15:0, anteiso-C15:0, C19:0, C20:0, and C20:0, anteiso-C15:0, iso-C14:0, 9,10-hexyl-C17:0, respectively. C16:1c7 was closely associated with C13:0, C17 FA (C17:0 and 9, 10-hexyl-C17:0), C16:1c9, C20:5n3 and C20:4n6. In conclusion, these data will be informative for the study on the regulatory mechanism of milk FA formation in yaks.

Key words: Correlations, fatty acid profile, lactation periods, Qinghai-Tibetan plateau, yak.

INTRODUCTION

Yak is a species that lives in mountainous areas of Central Asia, mainly found in the highlands of the Nepalese Himalayas, Indian Kashmir, Tibet, Mongolia, and Bhutan (Neupaney et al., 2003; Dhanapati et al., 2003), at an altitude of 2,500 000 to 5,500 000 m (Shi et al., 2019; Zi et al., 2008). The total world population of yaks is estimated at around 14.214.7 million of which China has the largest number of yaks in the world with approximately 13 14 million that comprises > 93.795% of all planet yaks (Sheng-Hua et al., 2013; Dong et al., 2007). Yak milk is a component of the diet in those areas contains 16.5 ± 2.8116.9-17.7% dry matter (DM), 4.90-5.39% protein, 5.53-7.28.8% fat, 4.9 ± 0.61 4.5-5.0% lactose, and 5.4 ± 2.43%0.8-0.9% minerals (Shi et al., 2019; Or-Rashid et al., 2008), thus is considered to be a naturally concentrated milk (Ren et al., 2014), accounting for 15 to 32% of a herder's daily fat intake (Liu et al., 2011). Considering the importance of fatty acids (FA) in affecting cheese quality, which is the main product of yak milk in Qinghai-Tibetan plateau

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(Liu et al., 2011; Gottardo et al., 2017), it will be of great value to determinate the FA composition of yak milk.

Milk fat of yak at very high altitudes is richer in polyunsaturated FA (PUFA) (Gottardo et al., 2017; Nikkhah, 2011). The amounts of conjugated linoleic acid (CLA) cis-9, trans-11 in yak cheese has been found to be 4.2 times greater than that in cow cheese (Jiang et al., 2007; Or-Rashid et al., 2008). Eicosapentaenoic acid (C20:5n3, EPA) and docosahexaenoic acid (C22:6n3, DHA)(Gottardo et al., 2017; Liu et al., 2011) are recognized as playing an essential role in human health, and are particularly important for the proper function of the brain, heart, and retina (Simopoulos, 1991; Kris-Etherton et al., 2003; Din et al., 2004; 1991). The greater content of low molecular weight volatile acids (Li et al., 2010) might prevent some human metabolic illness (Williams, 2000; Haenlein, 2004). Although the milk yield of yak has been reported (147 - 487 200-300 kg) and the lactation duration is just 150-200 days daily milk yield is commonly 1.5-2 kg (Dong et al., 2007; Zhou et al., 2014), considering the milk FA composition in each month varies a lot with the change of diet in dairy cow (Palmquist et al., 2006), determining the specific FA profile alteration across the lactation cycle may be beneficial for the utilization of yak milk.

The synthesis of FA are regulated by complex networks (Bionaz and Loor, 2008), results in the close association between different FA compositions (Samková and Kalač, 2021) (Maroteau et al., 2014). Many genes have reported to be responsible for the synthesis of different parts of FA (Stergiadis et al., 2020), such as short and medium chain FA (SMFA) (Zhu et al., 2014), monounsaturated FA (MUFA) (Shi et al., 2013), PUFA (Park et al., 2009) and very long chain FA (VLFA) (Matsuzaka et al., 2007). The milk fatty acids based on genetic and herd parameters was characterized in cow. Although the pathway leading to the formation of FA will not differ between yaks and cows, concentrations in milk fat are probably different. Investigate the exact association among yak milk FA compositions may enhance our understanding about the formation in yaks. The objective of present study was to investigate the FA profile of yak milk across the lactation cycle, and to identify the correlation among yak milk FA compositions. These data may be better for understanding the lactation physiology of yak, and facilitating the utilization of yak milk.

MATERIALS AND METHODS

Animals and sampling

The Animal Care and Use Committee of the Southwest University for Nationalities approved all procedures and experiments (Ministry of Science and Technology, China, revised in June 2004). Sixty-one healthy, similar weight, 4-7 years old, 3-6 parity *Maiwa* yaks with unrelated background were selected randomly from Hongyuan County, Sichuan province, in southwest of China (with the altitude over 3500 m) (Figure 1). The yaks graze on natural pasture all year around without irrigation, fertilizer, or other changes to the pasture. The experiment was conducted from May to November 2013. The samples for each animal were collected separately at 1, 2, 3, 4, 5, 6, 7, 15, 30, 60, 120 and 180 days postpartum. The yaks were hand milked once daily between 7 am to 8 am. About 100 ml milk samples (divided into 2 tubes after mixture) for each animal were then transferred using ice boxes from the farm to the laboratory in 6 h, and stored at −80°C until analysis.

Fatty acid extraction and analyses

The milk FA were extracted and methyl-esterified protocol was that 2 ml milk was completely mixed with 50 ml chloroform/methanol (2:1) solution (Bligh and Dyer, 1959; Folch et al., 1957). After being centrifuged at 3000 rpm for 5 min, the solution was transferred into a new tube and mixed with 10 ml 20% NaCl following by violent shock. Folch solution (chloroform:methanol:water/3:47:48) was then used for washing the tube wall. After standing for 5 min, the supernatant was removed. A total of 5 ml 0.5 M NaOH-CH_3OH was supplemented into the sample for methylation at 60°C for 30 min. Nitrogen gas was then used for evaporating the solution. 5 ml 14% BF_3-CH_3OH solution was added to the sample following standing at 80°C for 5 min. After the sample was cooled down to room temperature, 8 ml hexane was supplemented and shocked. The saturated NaCl solution was added until hierarchy. Following standing, the supernatant was transferred to a new tube. $CuSO_4$ was used for removing the water, and the supernatant was then used for gas chromatography-mass spectrometry (GC-MS, Agilent 7890A/5970C) analysis equipment with a HP88 column (60 m x 250 µm x 0.2 µm). Approximately 1 µl methylated lipid samples was injected into the GC system with 1:30 split ratio. The temperature of injector was 280°C. The initial temperature of the column was 120°C, which was temperature-programmed at 8°C/min to 145°C, continued to increase to 220°C at 3°C/min and maintained at 220°C for 3 min. The speed of carrier gas was 1 ml/min. The ionization mode was EI, with the ionization energy of 70 eV with 35 to 500 amu mass number. The temperature of interface, ion source and quadrupole rod were 280, 200 and 100°C respectively. The solvent delay was 3 min.

Statistical analyses

The analysis was carried out in duplicates and the data generated was expressed as mean ± standard deviation. Data obtained were statistically analysed by one-way ANOVA using the Statistical Package for the Social Sciences, version 22.0 (SPSS, Chicago, IL, USA), for significant F-statistics. If the overall F-test was significant ($P < 0.05$), a Fischer's T-test was performed to significant differences. The correlation analysis among different FA was calculated by Pearson method (2-tailed) using SPSS 22.0. The $|r| \geq 0.65$ with $P < 0.05$ was defined as correlated significantly.

RESULTS

Differences in milk saturated fatty acids across lactation cycle

A total of 38 FA and groups were detected. The FA individuals shorter than C10:0 could not be quantified due to solvent delay. Table 1 shows the fat content and the major FA profile of milk from primiparous yaks across the lactation cycle. The most abundant FA in yak milk were C16:0, C18:1c9, C18:0 and C14:0 across the lactation cycle, consistent with goat, sheep and cow (Markiewicz-Kęszycka et al., 2013). In detail, the relative content of C16:0 in yak milk had the highest content level at 2 days, and was decreased in a fluctuation model until 120 days postpartum, however

Figure 1. Map showing the sample collection site of Longri Seed Storage Chang, Hongyuan County, Sichuan Province. Source: AMAP software

with a sharp increase at 180 days to the similar level as 1 day postpartum. An inverse trend was observed in the relative content of C18:0, which increased in a fluctuation model, peaked at 7 and 120 days postpartum, with a sharp decrease at 180 days. The relative content of C14:0 was continually decreased across the whole lactation periods except a slight increase at 180 days. Considering that the relative content of FA compositions was seriously affected with the decrease of milk yield in the late lactation period, however, different results were observed at 180 days compared with the previous data (except C16:0) (Liu et al., 2011). Although the discrepancy of breeds, grazing model and pasture may result in different FA profile, the exact reason underlying the divergence remains unknown.

In ruminants, about one-half of milk FA are synthesized de novo (Frutos et al., 2020; Bauman and Davis, 1974), mainly including short- and medium- chain FA (C4-C14) and a portion of 16-carbon FA. Consistent with the data in Gannan yak (Liu et al., 2011), the relative content of C10:0 and C12:0 increased continually until 6 days, and turned down till the end of the lactation cycle. Following a stable period, the relative content of C17:0 was increased significantly at 120 days, however, with no significant change in 9,10-hexyl-C17:0, which was proved to increase cyclooxygenase activity in human. For the LCFA, no significant change was observed in C20:0 and C22:0 except for the significant increase after 30 days postpartum.

In milk fat odd branched-chain fatty acids (OBCFA) are important bioactive component due to their essential role in the gut and potential activity against human breast cancer cells (Gomez-Cortes et al., 2017). In the present study, similar as in dairy cow (Gomez-Cortes et al., 2017), C15:0, C17:0, anteiso-C15:0, iso-C15:0 and anteiso-C17:0 are most abundant OBCFA in yak milk fat. Differently, while a continue increase trend was observed in the content of C15:0 across the lactation cycle, the content of C17:0 decreased continually during colostrum period and increased continually till the end of lactation. However, we did not find iso-C17:0 in the present study. Besides, we also found 9, 10-hexyl-C17:0, the main cyclopropane FA in human adipose tissue and serum, in yak milk, with a continually decrease during colostrum period and turned up till the end of lactation. This may indicate the variation of cyclooxygenase activity during lactation in yaks (Sledzinski et al., 2013).

Differences in milk unsaturated fatty acids across lactation cycle

The C18:2t9t12 was the most abundant FA among the 3 kinds of linoleic acids observed in the present study.

Table 1. The fat content (g/100 g) and the major FA profile (% in total fatty acid methyl esters, g/100 g total FAs) of milk from yaks across the lactation cycle.

Traits	0d	1d	2d	3d	4d	5d	6d	7d	15d	30d	120d	180d
C10:0	1.54±0.47^{a}	1.97±0.60^{c}	2.24±0.58^{d}	2.31±0.63^{de}	2.32±0.57^{de}	2.46±0.70^{e}	2.27±0.64^{de}	2.01±0.55^{c}	1.82±0.35^{bc}	1.66±0.36^{a}	1.53±0.29^{ab}	1.60±0.26^{a}
C10:1t4	0.03±0.02^{a}	0.04±0.02^{b}	0.05±0.03^{bc}	0.06±0.03^{cd}	0.06±0.02^{cd}	0.07±0.03^{de}	0.06±0.03^{d}	0.07±0.03^{de}	0.08±0.04^{ef}	0.09±0.04^{fg}	0.09±0.04^{fg}	0.10±0.05^{g}
C12:0	1.68±0.34^{a}	1.97±0.48^{b}	2.16±0.48^{c}	2.17±0.49^{c}	2.20±0.50^{c}	2.30±0.61^{c}	2.13±0.57^{c}	1.87±0.49^{b}	1.65±0.27^{a}	1.62±0.39^{a}	1.57±0.46^{a}	1.65±0.29^{a}
C13:0	0.03±0.02^{a}	0.04±0.01^{ab}	0.04±0.02^{ab}	0.04±0.01^{abc}	0.04±0.01^{abc}	0.04±0.01^{bcd}	0.04±0.01^{abcd}	0.04±0.01^{abc}	0.04±0.02^{abc}	0.04±0.02^{cd}	0.04±0.02^{cd}	0.05±0.03^{d}
iso-C14:0	0.10±0.05^{a}	0.11±0.03^{a}	0.12±0.04^{ab}	0.13±0.04^{b}	0.13±0.03^{b}	0.14±0.05^{b}	0.13±0.04^{b}	0.13±0.04^{b}	0.14±0.05^{b}	0.19±0.10^{c}	0.21±0.07^{d}	0.21±0.07^{cd}
C14:0	10.32±1.89^{d}	9.67±1.32^{bc}	10.02±1.44^{cd}	9.81±1.26^{bc}	9.75±1.34^{bc}	9.73±1.35^{bc}	9.36±1.17^{b}	8.87±1.06^{a}	8.51±0.69^{a}	8.55±1.07^{a}	8.41±1.03^{a}	8.81±0.69^{a}
Iso-C15:0	0.28±0.11^{a}	0.29±0.08^{ab}	0.29±0.10^{a}	0.32±0.10^{bcd}	0.33±0.08^{bcd}	0.33±0.09^{cd}	0.32±0.09^{bcd}	0.32±0.08^{bcd}	0.30±0.07^{abc}	0.35±0.07^{d}	0.46±0.06^{e}	0.46±0.12^{e}
Anteiso-C15:0	0.66±0.21^{a}	0.69±0.19^{a}	0.72±0.24^{a}	0.80±0.23^{b}	0.82±0.19^{b}	0.82±0.20^{b}	0.82±0.18^{b}	0.91±0.17^{c}	0.96±0.17^{c}	1.06±0.11^{d}	1.18±0.12^{e}	1.18±0.19^{e}
C15:0	1.03±0.21^{a}	1.13±0.17^{bc}	1.08±0.21^{ab}	1.12±0.17^{bc}	1.13±0.15b^{c}	1.12±0.17^{bc}	1.13±0.16^{bc}	1.15±0.13^{bc}	1.15±0.15^{c}	1.24±0.11^{d}	1.35±0.19^{e}	1.34±0.21^{e}
C16:0	36.01±5.51^{de}	35.07±3.95^{cd}	36.81±4.14^{e}	35.77±4.30^{de}	35.18±3.74^{cd}	36.01±3.99^{de}	35.58±3.94^{cde}	34.03±3.54^{bc}	34.41±3.06^{bcd}	33.31±4.14^{ab}	32.28±2.60^{a}	34.87±3.90^{cd}
C16:1c9	0.06±0.06^{bc}	0.04±0.04^{ab}	0.02±0.03^{a}	0.03±0.04^{a}	0.03±0.04^{a}	0.04±0.05^{ab}	0.04±0.05^{ab}	0.07±0.08^{cd}	0.10±0.08^{e}	0.09±0.06^{de}	0.07±0.05^{cd}	0.03±0.04^{a}
C16:1c7	0.16±0.12^{d}	0.15±0.10^{cd}	0.10±0.09^{a}	0.11±0.08^{a}	0.11±0.08^{abc}	0.12±0.08^{abc}	0.12±0.09^{abc}	0.11±0.10^{abc}	0.13±0.09^{abcd}	0.14±0.09^{bcd}	0.13±0.10^{abcd}	0.11±0.09^{ab}
C16:1t2	0.76±0.33^{cd}	0.74±0.29^{bcd}	0.60±0.30^{a}	0.62±0.29^{a}	0.63±0.27^{ab}	0.61±0.28^{a}	0.59±0.24^{a}	0.67±0.22^{abc}	0.83±0.33^{de}	0.86±0.35^{de}	0.94±0.34^{e}	1.11±0.48^{f}
Anteiso-C17:0	0.32±0.16^{bcd}	0.32±0.14^{abcd}	0.26±0.14^{a}	0.29±0.14^{abc}	0.30±0.12^{abcd}	0.27±0.14^{ab}	0.27±0.13^{ab}	0.26±0.12^{a}	0.27±0.12^{a}	0.27±0.11^{ab}	0.35±0.17^{d}	0.35±0.16^{cd}
C17:0	0.97±0.34^{c}	0.95±0.29^{bc}	0.78±0.28^{a}	0.85±0.27^{ab}	0.85±0.20^{ab}	0.84±0.25^{ab}	0.85±0.23^{ab}	0.79±0.25^{a}	0.80±0.25^{a}	0.85±0.23^{ab}	1.08±0.24^{d}	1.11±0.41^{d}
9,10-hexyl-C17:0	0.13±0.10^{bc}	0.11±0.08^{b}	0.07±0.06^{a}	0.08±0.05^{a}	0.08±0.05^{a}	0.08±0.06^{a}	0.08±0.05^{a}	0.07±0.05^{a}	0.09±0.05^{a}	0.13±0.10^{bc}	0.15±0.08^{c}	0.18±0.09^{d}
C18:0	16.48±3.51^{a}	17.62±2.78^{bc}	17.83±2.94^{bcd}	18.49±2.79^{cde}	18.78±2.79^{de}	18.39±2.78^{cde}	19.04±2.21^{e}	19.24±2.04^{e}	18.71±1.87^{de}	18.68±2.37^{de}	19.12±1.94e	16.96±2.03^{ab}
C18:1t8	3.30±1.40^{b}	3.83±1.49^{c}	3.88±1.33^{c}	4.33±1.37^{cde}	4.44±1.34^{de}	4.50±1.34^{e}	4.87±1.35^{ef}	6.00±1.68^{g}	6.11±1.49^{g}	5.13±1.63^{f}	3.92±1.25^{cd}	2.71±0.78^{a}
C18:1c9	22.08±3.65^{de}	21.32±4.01^{d}	19.59±3.28^{bc}	19.03±2.91^{abc}	18.76±3.18^{ab}	18.37±3.12^{a}	18.44±3.15^{a}	19.36±2.56^{abc}	19.90±1.70^{c}	21.56±2.29^{d}	22.35±1.03^{de}	23.10±1.75^{e}
C18:1t11	0.47±0.22^{abc}	0.48±0.17^{c}	0.40±0.20^{a}	0.43±0.17^{abc}	0.45±0.16^{abc}	0.40±0.16^{a}	0.42±0.14^{abc}	0.45±0.16^{abc}	0.44±0.15^{abc}	0.44±0.16^{abc}	0.47±0.15^{bc}	0.41±0.17^{ab}
C18:1t13	0.11±0.09^{a}	0.11±0.08^{ab}	0.10±0.09^{a}	0.12±0.09^{ab}	0.15±0.09^{bcd}	0.13±0.08^{ab}	0.13±0.08^{abc}	0.16±0.09^{cde}	0.18±0.09^{def}	0.20±0.11^{f}	0.20±0.10^{ef}	0.17±0.10^{cde}
C18:2c9c12	0.21±0.12^{a}	0.22±0.11^{ab}	0.19±0.12^{a}	0.24±0.12^{abc}	0.27±0.11^{bcd}	0.26±0.12^{bcd}	0.27±0.13^{bcd}	0.28±0.14^{cd}	0.28±0.12^{cde}	0.31±0.12^{de}	0.39±0.16^{f}	0.33±0.15^{e}
C18:2t9t12	1.25±0.45^{f}	1.11±0.35^{e}	0.90±0.33^{cd}	0.91±0.29^{cd}	0.96±0.24^{d}	0.88±0.28^{cd}	0.82±0.29^{bc}	0.73±0.26^{ab}	0.67±0.20^{a}	0.67±0.28^{a}	0.83±0.26^{bc}	0.62±0.16^{a}
C18:2t9c11	0.69±0.37^{a}	0.78±0.32^{abc}	0.74±0.38^{ab}	0.84±0.37^{abc}	0.87±0.36^{bcd}	0.87±0.34^{bcd}	0.91±0.33^{cd}	1.30±0.56^{fg}	1.46±0.53^{g}	1.34±0.51^{g}	1.16±0.47^{ef}	1.01±0.39^{de}
C18:2c9c15	0.01±0.02^{ab}	0.01±0.01^{ab}	0.01±0.01^{a}	0.01±0.02^{a}	0.01±0.02^{ab}	0.02±0.02^{ab}	0.01±0.02^{ab}	0.02±0.02^{ab}	0.02±0.03^{abc}	0.03±0.04^{bcd}	0.03±0.03^{cd}	0.04±0.10^{d}
C18:3n3	0.61±0.24^{b}	0.61±0.19^{b}	0.53±0.24^{ab}	0.58±0.21^{b}	0.60±0.21^{b}	0.57±0.19^{ab}	0.56±0.20^{ab}	0.54±0.20ab	0.49±0.15^{a}	0.60±0.21^{b}	0.80±0.24^{c}	0.60±0.19^{b}
C19:0	0.13±0.08^{a}	0.15±0.06^{abc}	0.13±0.07^{ab}	0.15±0.07^{abc}	0.17±0.06^{cd}	0.15±0.06^{abc}	0.16±0.06^{bc}	0.15±0.06^{abc}	0.15±0.06^{abc}	0.15±0.05^{abc}	0.18±0.07^{d}	0.17±0.08^{cd}
C20:0	0.20±0.11^{bc}	0.21±0.08^{bc}	0.19±0.11^{abc}	0.22±0.09^{bc}	0.22±0.08^{c}	0.20±0.08^{abc}	0.21±0.08^{bc}	0.18±0.06^{ab}	0.16±0.05^{a}	0.22±0.08^{bc}	0.40±0.18^{d}	0.44±0.23^{e}
C22:0	0.04±0.03^{a}	0.04±0.03^{a}	0.04±0.04^{a}	0.05±0.03^{a}	0.05±0.03^{a}	0.05±0.03^{a}	0.05±0.03^{a}	0.05±0.03^{a}	0.05±0.03^{a}	0.07±0.04^{b}	0.13±0.06^{c}	0.14±0.07^{c}
C20:4n6	0.03±0.03^{e}	0.03±0.03^{de}	0.02±0.03^{abcd}	0.03±0.02^{cde}	0.03±0.02^{cde}	0.03±0.02^{cde}	0.02±0.02^{bcde}	0.01±0.02^{ab}	0.01±0.01^{a}	0.02±0.03^{abc}	0.02±0.02^{abc}	0.03±0.05^{cde}
C20:5n3	0.05±0.04^{d}	0.05±0.05^{d}	0.03±0.03^{ab}	0.03±0.03^{abc}	0.04±0.03^{abcd}	0.04±0.03^{abcd}	0.03±0.03^{abc}	0.02±0.03^{a}	0.03±0.03^{ab}	0.04±0.04^{cd}	0.05±0.04^{d}	0.04±0.04^{bcd}
MCFA	15.67±1.90^{bc}	15.91±2.10^{bcd}	16.73±2.08^{d}	16.77±1.87^{d}	16.78±2.06^{d}	17.02±2.38^{d}	16.30±2.17^{c}	15.35±1.91^{ab}	14.68±1.17^{a}	14.68±1.55^{a}	14.88±1.57^{a}	15.35±1.12^{ab}
LCFA	83.87±2.08^{bcdef}	83.72±2.04^{a}	82.98±2.02^{abcd}	82.90±1.81^{abcd}	82.87±2.01^{abc}	82.66±2.34^{ab}	83.35±2.11^{abcde}	84.28±2.02^{cdef}	85.06±1.17^{f}	84.56±2.20^{ef}	84.39±1.48^{def}	83.87±1.54^{bcdef}
VLCFA	0.32±0.18^{abc}	0.33±0.16^{bc}	0.28±0.19^{abc}	0.33±0.16^{bc}	0.34±0.14^{c}	0.32±0.14^{abc}	0.32±0.14^{abc}	0.26±0.13^{ab}	0.25±0.12^{a}	0.35±0.16^{c}	0.62±0.27^{d}	0.65±0.29^{d}
SFA	70.15±3.97^{c}	70.39±3.91^{c}	72.83±3.88^{d}	72.62±3.92^{d}	72.36±3.53^{d}	73.18±3.93^{d}	72.60±3.97^{d}	70.15±3.73^{c}	69.26±2.86^{abc}	68.15±3.35^{a}	68.53±2.83^{ab}	69.56±2.46^{bc}

Table 1. Contd.

USFA	29.85±3.97^{b}	29.58±3.90^{b}	27.17±3.88^{a}	27.38±3.92^{a}	27.64±3.53^{a}	26.82±3.93^{a}	27.36±3.98^{a}	29.85±3.73^{b}	30.74±2.86^{bc}	31.73±3.27^{c}	31.35±2.73^{c}	30.45±2.45^{bc}
MUFA	26.98±3.56^{bc}	26.74±3.55^{b}	24.73±3.31^{a}	24.72±3.38^{a}	24.85±3.10^{a}	24.17±3.52^{a}	24.69±3.51^{a}	26.94±3.09^{b}	27.77±2.34^{bcd}	28.67±2.85^{d}	28.16±1.78^{cd}	27.91±1.79^{bcd}
PUFA	2.87±1.01^{bcd}	2.84±0.79^{bcd}	2.44±0.97^{a}	2.65±0.77^{ab}	2.76±0.72^{abcd}	2.65±0.70^{ab}	2.70±0.61^{abc}	2.91±0.90^{bcd}	3.02±0.74^{cde}	3.07±0.74d^{e}	3.30±0.95^{e}	2.64±0.75^{ab}

$^{a–g}$Different superscript letters indicate significant differences (P <0.05) within the same row. MCFA, medium-chain FA (sum of C10-C15); LCFA, long-chain FA (sum of C16-C22); VLCFA (sum of C20-C22); SFA, saturated FA (sum of C10:0, C12:0-C16:0, anteiso-C17:0 -C18:0, C19:0-C22:0); USFA, unsaturated FA (sum of C10:1n4, C16:1, C18:1, C18:2, C18:3, C20:4, C20:5); MUFA (sum of C10:1n4, C16:1, C18:1); PUFA (sum of C18:2, C18:3, C20:4, C20:5).
Source: Authors

Although there was no significant change for the total content of linoleic acids, C18:2t9t12 continually decreased during lactation periods. Different from our results that cis-9, trans-11 CLA and trans-10, cis-12 CLA were the main CLA in dairy cow (Harvatine et al., 2009; and Wang et al., 2012), dairy goat (Maroteau et al., 2014) and even in *Gannan yak* (Liu et al., 2011). A possible reason was that the GC system was not sufficient for distinguishing different CLA types only using individual cis-9, trans-11 CLA and trans-10, cis-12 CLA references in yak milk (Liu et al., 2011). In the present study, a GC-MS system was used for more reliable results by comparing the different types of CLA with the NIST mass spectra library (2009). Obviously, more research by GC-MSMS in yak milk is essential for confirming the results in the future.

Consistent with previous study in goat (Haile et al., 2016), dairy cow (Chilliard and Ferlay, 2004) and *Gannan yak* (Liu et al., 2011), the C18:1c9 was the most abundant USFA. Different with the fluctuation model of C16:0 and C18:0, the relative content of C18:1c9 was continually decreased during colostrum period and turned over after 5 days postpartum. Similar change model was observed in C16:1c9 and C16:1t2 which was the most abundant derivative of palmitic acid. In ruminants, stearoyl-coenzyme A desaturase (*SCD*) is a primary candidate gene for altering the proportion of SFA versus MUFA in milk with the palmitic acid and stearic acid as substrates (Shi et al., 2013). The relative content of MUFA C18:1c9, compared to the sum of C16:0 and C18:0, was significantly lower than that in dairy goat (Haile et al., 2016) and dairy cow (Cortes et al., 2010), may indicate a lower activity of SCD in yak milk. However, in *Gannan yak* the value of MUFA/SFA was much higher than that in dairy cow (Wang et al., 2012). Although different breeds of yak may be different in terms of milk fatty acid profile, obviously more controlled study is still needed for elucidating the discrepancy. We also observed small portion of ARA and EPA which presented the activity of fatty acid desaturase 1 (FADS1) (Park et al., 2009), bottomed at 3 and 9 /8 days postpartum, respectively.

Correlations between milk FA

Table 2 showed the FA correlations in Maiwa yak milk, while the Figures 1 and 2 showed the significant correlations. In general, C16:1c7, C18:1t11, C22:0 and iso-C15:0 correlated with most of the FA individuals. As expected, the FA de novo synthesized in mammary gland, including C10:0, C12:0, C14:0 and part of C16:0, significantly correlated with each other, and negatively correlated with C18:0 (r=-0.64). Close correlation was observed among C18 FAs, including C18:1t11, C18:1t13, C18:2t9t12, ALA, and also anteiso-C17:0 (Ntambi and Miyazaki, 2004). While the C16:1c7 was then converted to C18:1c7, C18:1c9 was mainly desaturated from C18:0 (Green et al., 2010). Although C18:2t9c11 was not correlated with the individuals, there was a significantly negative correlation with the total content of SFA, supporting the results of MFD effect on de novo fatty acid synthesis (Piperova et al., 2000).

Close correlations were observed among C15 FAs, C20:0 and C22:0, indicating that anteiso-C15:0, iso-C15:0 and C15:0 may have the same source (Gomez-Cortes et al., 2017). The close correlation between iso-C15:0, as well as 9, 10-hexyl-C17:0 and C17:0, and the total content of VLCFA may indicate their involvement in FA elongation. The C22:0 may be the elongation product of C20:0 in yak milk. Iso-C14:0 was correlated with C13:0 instead of C14:0, indicating that they may share similar source in yak milk (Gomez-Cortes et al., 2017).

DISCUSSION

Although the fatty acid profile of yak milk from the Qinghai-Tibetan plateau in different seasons has been identified (Liu et al., 2011), the present study is novel in that we investigated the variation of milk FA profile across the whole lactation cycle, and analyzed the correlation between FA individuals in grazed yak without the transfer from summer pasture to winter

Table 2. Pearson correlation of fatty acids in yak milk.

Traits	C10:0	C10:1t4	C12:0	C13:0	iso-C14:0	C14:0	iso-C15:0	anteiso-C15:0	C15:0	C16:0	C16:1c9	C16:1c7	C16:1t2	anteiso-C17:0	C17:0	9,10-hexyl-C17:0
C10:0	1															
C10:1t4	0.20	1														
C12:0	0.90	0.16	1													
C13:0	0.06	0.64	0.05^{NS}	1												
iso-C14:0	-0.23	0.60	-0.28	0.74	1											
C14:0	0.62	-0.06^{NS}	0.73	-0.12	-0.38	1										
Iso-C15:0	-0.31	0.44	-0.23	0.48	0.61	-0.45	1									
Anteiso-C15:0	-0.40	0.54	-0.38	0.41	0.61	-0.56	0.84	1								
C15:0	-0.39	0.21	-0.39	0.34	0.53	-0.60	0.78	0.82	1							
C16:0	0.43	-0.04^{NS}	0.35	-0.18	-0.23	0.63	-0.57	-0.55	-0.58	1						
C16:1c9	-0.13	0.38	-0.19	0.61	0.43	-0.23	0.20	0.27	0.17	-0.21	11					
C16:1c7	-0.19	0.35	-0.18	0.71	0.47	-0.21	0.32	0.25	0.22	-0.28	0.77	1				
C16:1t2	-0.44	0.18	-0.37	-0.05^{NS}	0.14	-0.38	0.47	0.58	0.51	-0.33	-0.06^{NS}	0.03^{NS}	1			
Anteiso-C17:0	-0.40	-0.10	-0.30	-0.01^{NS}	0.13	-0.42	0.53	0.49	0.64	-0.58	-0.06^{NS}	0.11	0.67	1		
C17:0	-0.39	0.27	-0.32	0.52	0.54	-0.39	0.64	0.48	0.56	-0.48	0.39	0.67	0.26	0.45	1	
9,10-Hexyl-C17:0	-0.42	0.33	-0.37	0.48	0.54	-0.35	0.56	0.52	0.48	-0.36	0.44	0.67	0.48	0.47	0.73	1
C18:0	-0.10	-0.19	-0.11	0.01^{NS}	0.03^{NS}	-0.45	0.21	0.12	0.24	-0.64	0.01^{NS}	-0.01^{NS}	-0.25	0.11	0.09	-0.12
C18:1t8	-0.02^{NS}	-0.06^{NS}	-0.10	0.07^{NS}	-0.06^{NS}	-0.31	0.01^{NS}	0.21	0.21	-0.36	0.35	0.08	-0.05^{NS}	0.09	-0.20	-0.11
C18:1c9	-0.71	-0.05^{NS}	-0.65	-0.10	0.20	-0.53	0.27	0.31	0.27	-0.48	-0.05^{NS}	0.11	0.46	0.36	0.41	0.38
C18:1t11	-0.33	-0.02^{NS}	-0.18	0.10	0.02^{NS}	-0.37	0.48	0.41	0.52	-0.67	0.14	0.30	0.57	0.79	0.45	0.47
C18:1t13	-0.23	0.32	-0.10	0.30	0.22	-0.39	0.55	0.57	0.50	-0.64	0.32	0.32	0.41	0.54	0.32	0.39
C18:2c9c12	-0.29	0.31	-0.25	0.32	0.37	-0.41	0.56	0.58	0.48	-0.46	0.26	0.27	0.45	0.44	0.34	0.44
C18:2t9t12	-0.20	-0.29	-0.04^{NS}	-0.01^{NS}	-0.15	0.02^{NS}	0.12	-0.08	0.12	-0.29	-0.05^{NS}	0.29	0.22	0.49	0.42	0.33
C18:2t9c11	-0.35	0.22	-0.39	0.14	0.20	-0.55	0.35	0.62	0.52	-0.50	0.42	0.19	0.52	0.41	0.12	0.26
C18:2c9c15	-0.11	0.48	-0.11	0.71	0.67	-0.12	0.35	0.24	0.17	-0.03^{NS}	0.70	0.63	-0.02^{NS}	0.02^{NS}	0.38	0.43
C18:3n3	-0.29	0.13	-0.13	0.24	0.24	-0.28	0.60	0.46	0.52	-0.56	0.13	0.29	0.43	0.60	0.54	0.46
C19:0	-0.20	0.26	-0.13	0.47	0.39	-0.38	0.70	0.58	0.64	-0.63	0.31	0.47	0.37	0.60	0.64	0.62
C20:0	-0.35	0.18	-0.33	0.22	0.49	-0.41	0.68	0.57	0.65	-0.41	0.04^{NS}	0.20	0.54	0.61	0.60	0.64
C22:0	-0.33	0.45	-0.33	0.47	0.71	-0.42	0.73	0.65	0.62	-0.36	0.24	0.34	0.43	0.41	0.63	0.71

Table 2. cont'd

C20:4n6	0.05^{NS}	0.30	0.06^{NS}	0.62	0.46	0.04^{NS}	0.18	0.02^{NS}	0.02^{NS}	-0.01^{NS}	0.40	0.67	-0.07^{NS}	0.04^{NS}	0.50	0.52
C20:5n3	-0.07^{NS}	0.38	-0.01^{NS}	0.66	0.50	-0.06^{NS}	0.30	0.17	0.15	-0.18	0.57	0.72	0.02^{NS}	0.10	0.56	0.59
MCFA	0.84	0.21	0.92	0.11	-0.18	0.88	-0.19	-0.32	-0.37	0.48	-0.16	-0.14	-0.33	-0.33	-0.29	-0.33
LCFA	-0.80	-0.26	-0.87	-0.16	0.11	-0.82	0.11	0.25	0.29	-0.45	0.11	0.08	0.29	0.28	0.20	0.16
VLCFA	-0.31	0.35	-0.28	0.47	0.64	-0.37	0.69	0.56	0.59	-0.38	0.25	0.44	0.43	0.49	0.70	0.77
SFA	0.74	-0.02^{NS}	0.69	-0.05^{NS}	-0.22	0.72	-0.44	-0.56	-0.53	0.75	-0.25	-0.28	-0.59	-0.57	-0.44	-0.52
USFA	-0.74	0.02^{NS}	-0.69	0.05^{NS}	0.22	-0.71	0.43	0.56	0.53	-0.75	0.25	0.29	0.60	0.58	0.44	0.52
MUFA	-0.75	-0.02^{NS}	-0.71	-0.01^{NS}	0.19	-0.71	0.37	0.51	0.46	-0.71	0.20	0.22	0.54	0.50	0.40	0.46
PUFA	-0.41	0.15	-0.32	0.27	0.24	-0.45	0.53	0.56	0.57	-0.62	0.38	0.45	0.55	0.65	0.50	0.58

Traits	**C18:0**	**C18:1t8**	**C18:1c9**	**C18:1c11**	**C18:1c13**	**C18:2c9c12**	**C18:2t9t,12**	**C18:2t9c11**	**C18:2c9c15**	**C18:3n3**	**C19:0**	**C20:0**	**C22:0**	**C20:4n6**	**C20:5n3**	**MCFA**	**LCFA**	**VLCFA**	**SFA**	**USFA**	**MUFA**
C10:0																					
C10:1t4																					
C12:0																					
C13:0																					
iso-C14:0																					
C14:0																					
iso-C15:0																					
anteiso-C15:0																					
C15:0																					
C16:0																					
C16:1c9																					
C16:1c7																					
C16:1t2																					
anteiso-C17:0																					
C17:0																					
9,10-hexyl-C17:0																					
C18:0	1																				
C18:1t8	0.40	1																			
C18:1c9	-0.02^{NS}	-0.30	1																		
C18:1t11	0.22	0.28	0.26	1																	
C18:1t13	0.33	0.34	0.08	0.72	1																

Table 2. cont'd

C18:2c9c12	0.11	0.29	0.11	0.51	0.58	1															
C18:2t9t12	-0.00^{NS}	-0.21	0.20	0.58	0.20	0.15	1														
C18:2t9c11	0.08	0.70	0.14	0.50	0.54	0.56	-0.07	1													
C18:2c9c15	-0.14	0.03^{NS}	-0.06^{NS}	0.02^{NS}	0.18	0.34	-0.03^{NS}	0.20	1												
C18:3n3	0.21	-0.01^{NS}	0.18	0.71	0.63	0.56	0.66	0.27	0.15	1											
C19:0	0.33	0.20	0.11	0.68	0.60	0.55	0.38	0.38	0.23	0.67	1										
C20:0	0.11	-0.19	0.30	0.42	0.37	0.52	0.25	0.21	0.26	0.59	0.61	1									
C22:0	0.08	-0.19	0.28	0.29	0.41	0.50	0.08	0.22	0.47	0.52	0.59	0.87	1								
C20:4n6	-0.14	-0.23	-0.01^{NS}	0.15	0.13	0.13	0.33	-0.13	0.55	0.24	0.35	0.20	0.37	1							
C20:5n3	-0.06^{NS}	-0.12	0.06^{NS}	0.23	0.31	0.25	0.31	0.03^{NS}	0.58	0.38	0.45	0.29	0.48	0.83	1						
MCFA	-0.32	-0.21	-0.65	-0.27	-0.20	-0.26	-0.05^{NS}	-0.42	-0.10	-0.16	-0.16	-0.28	-0.25	0.11	0.03^{NS}	1					
LCFA	0.34	0.23	0.64	0.23	0.14	0.19	-0.03^{NS}	0.41	-0.08	0.09	0.11	0.16	0.15	-0.16	-0.10	-0.97	1				
VLCFA	0.06^{NS}	-0.22	0.27	0.39	0.40	0.51	0.25	0.17	0.47	0.59	0.66	0.92	0.94	0.50	0.58	-0.21	-0.01^{NS}	1			
SFA	-0.15	-0.26	-0.78	-0.62	-0.47	-0.46	-0.25	-0.65	-0.07^{NS}	-0.41	-0.42	-0.36	-0.32	0.03^{NS}	-0.12	0.74	-0.54	-0.32	1		
USFA	0.15	0.27	0.78	0.63	0.47	0.46	0.25	0.65	0.07^{NS}	0.41	0.43	0.36	0.32	-0.02^{NS}	0.12	-0.73	0.55	0.32	-0.99	1	
MUFA	0.14	0.21	0.84	0.52	0.37	0.36	0.14	0.57	0.11	0.27	0.31	0.30	0.26	-0.08	0.05^{NS}	-0.76	0.62	0.24	-0.98	0.98	1
PUFA	0.11	0.37	0.21	0.81	0.67	0.72	0.56	0.70	0.40	0.80	0.69	0.49	0.43	0.24	0.38	-0.35	0.04^{NS}	0.50	-0.66	0.67	0.52

NS, Not significantly different. MCFA, medium-chain FA (sum of C10-C15); LCFA, long-chain FA (sum of C16-C22); VLCFA (sum of C20-C22); SFA, saturated FA (sum of C10:0, C12:0-C16:0, anteiso-C17:0 -C18:0, C19:0-C22:0); USFA, unsaturated FA (sum of C10:1n4, C16:1, C18:1, C18:2, C18:3, C20:4, C20:5); MUFA (sum of C10:1n4, C16:1, C18:1); PUFA (sum of C18:2, C18:3, C20:4, C20:5).
Source: Authors

pasture. This was predicted to be the main reason for the discrepancy between our results and Gannan yaks (Liu et al., 2011). In addition, the innovative use of GC-MS, instead of the GC equipment (Or-Rashid et al., 2008; Liu et al., 2011), guaranteed the believable qualitative results of FA composition in yak milk. Although more confirming studies remain needed, these data will be informative for the study of milk FA profile in the future.

We also recognized the limitation of relative quantitative of FA composition. However, this method was used for milk FA profile study in goat (Haile et al., 2016). We also had investigated the variation trend of yak milk FA composition across the whole lactation cycle, and built the correlation among different FA individuals based on a relative quantitative method, which were the main objectives in the present study. Although an absolute quantitative method may be beneficial for detecting the exact content of FA individuals in yak milk, our data also will be informative for understanding the yak milk FA formation regulation.

Another limitation was that we gave a possible that SCD and EVOVLs may be involved in the correlation of FA desaturation and elongation, especially for MUFA and VLCFA, we did not detect the expression of these genes. Obviously, if we could detect the expression of the genes associated with milk fat formation in the mammary gland (Zhu et al., 2014, 2015, 2016), and build their correlation with the FA individuals will enhance our understanding about the regulatory mechanism of FA profile formation in yak milk in the future.

Conclusion

This work studied the variation of milk FA profile across the lactation cycle in yak milk grazed at 3,500 m above sea level on the Qinghai-Tibetan plateau. The most abundant FA in yak milk are C16:0, C18:1c9, C18:0 and C14:0, and are varied significantly along with the lactation periods. Close correlations existed among different FA individuals, in which C18:1t11, iso-C15:0, C22:0 and C16:1c7 are at the central positions, indicating that they may paly important role

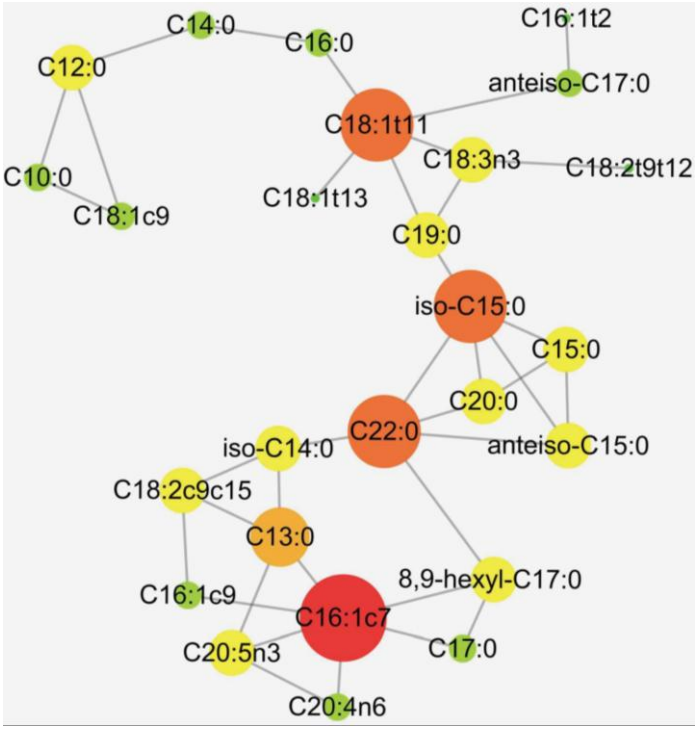

Figure 2. Significant correlation of fatty acids in yak milk. The correlation network was performed using the software of Cytoscape 3.1. Bigger node size was associated with more of FA individuals. The color of the node was mapped according to eccentricity. The $|r| \geq 0.65$ with $P < 0.05$ was defined as correlated significantly.
Source: Authors

in FA profile formation in yak milk. These data will be informative for the study about the regulatory mechanism underlying the milk FA formation in yak.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

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Analgesic and anti-inflammatory activities of propolis obtained from Gboko, Nigeria

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The use of propolis for the treatment of various ailments in which inflammation and pain is involved is cross-cultural. This report was designed to investigate the analgesic and anti-inflammatory activities of propolis obtained from Gboko, Benue State using hexane, ethyl acetate, and ethanol solvents. Propolis sample obtained from beehives was homogenized, extracted and dried at 40°C. The dry extract was prepared in a solution of olive oil for pharmacological and toxicological tests. Acute toxicity of propolis in varying polarity was determined using albino rats. Oedema volume of albino rat's paw was used to determine the anti-inflammatory activity of the sample. Analgesic property of the sample was investigated by measuring the number of acetic acid induced writhing in albino rats. Hexane, ethyl acetate, and ethanol extracts of propolis were non-toxic at 1,000 mg/kg bodyweight. During anti-inflammatory investigation, sample at 300 and 200 mg/kg bodyweight showed better activity than the standard drug, indomethacin. Best anti-inflammatory activity was observed with ethanol fraction at 300 mg/kg bodyweight. Best analgesic activity (100%) was recorded at 200 mg/kg in hexane. These results show that both non-polar and polar extracts of propolis have anti-inflammatory and analgesic activities at various dosages.

Key words: Propolis, anti-inflammatory, analgesic, acetic acid, diclofenac, indomethacin, Nigeria, polarity.

INTRODUCTION

A natural product is a chemical compound or substance produced by living organisms such as plants, animals, microbes, algae, and marine sponges. The term natural product has also been extended for commercial purposes to refer to cosmetics, dietary supplements, and foods produced from natural sources without added artificial ingredients. Several reasons such as knowledge of composition, structure and bioactivities of natural products necessitate the study of Natural Products (Nande and Igoli, 2017). Increasing evidence suggest that majority of the bioactive phytochemical components in plants impart physiological activities and may offer a variety of health benefits such as antioxidant, antibacterial, anti-inflammatory, and anticancer (Ipav et al., 2018) or anti-venom (Tor-Anyiin et al., 2015). Propolis is a natural product derived from plant resins and collected by honeybees (workers) to be used as glue and as draught-extruder for beehives (Khalil, 2006). Recent foray into

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bee propolis research has brought remarkable health benefits thereby making propolis a potential source for development of new drugs (Bankova et al., 2000). It has been reported that propolis contain at least 200 compounds with more than 100 being present in any given sample (Greenaway et al., 1999). These include fatty and phenolic acids and esters, substituted phenolic esters, bioflavonoids (flavones, flavanones, flavonols and others), terpenes, steroids, aromatic aldehydes and alcohols, and derivatives of sesquiterpenes, naphthalene and stilbenes (Marcucci et al., 1996).

Several research studies have corroborated the claim that, chemical composition and bioactivities of propolis depend on the phyto-geographical characteristics of the site of location which then varies with vegetation belts, countries, states and locality. Accordingly, these seasonal variations can influence the biosynthesis of plant metabolites and consequently affect the resin that is secreted by plants, which is used by the honeybees to produce propolis (Teixeira et al., 2008). According to Babatunde et al. (2015) and Khalil (2006), despite its sensitivity to geographical locations and the fact that constituent compositions differ according to each area, most propolis samples contain 50% resin, 30% wax, 10% essential oils, 5% pollen and 5% of other organic compounds in their chemical composition.

Due to the promise propolis holds, it has attracted researchers' interest in the last decades because of its several biological and pharmacological properties including anti-inflammatory, analgesic, anticancer and antimicrobial activities (Christov et al., 2005). Several reports of the biological activities of propolis have been made in literature including antioxidant (Frozza et al., 2013), antimicrobial (Bispo-Junior et al., 2012), anticancer (Li et al., 2008), anti-inflammatory (Bueno-Silva et al., 2016; Cavendish et al., 2015), cytotoxicity (Alencar et al., 2007), repair of wounds (Batista et al., 2012) and antinociceptive (Cavendish et al., 2015) properties. Few reports are found on the propolis of Nigerian origin and none from Gboko.

In this paper, we report the analgesic and anti-inflammatory effect of extracts of propolis obtained from Gboko, Benue State using solvents of varying polarity; hexane, ethyl acetate and ethanol.

MATERIALS AND METHODS

Collection of propolis sample

Propolis sample was obtained from bee keepers in Gboko and the vegetation surrounding the hives was noted as *Maranthes polyandra, Detarium microcarpum* and *Burkea africana*.

Preparation of propolis extract

The homogenized propolis sample was placed in the thimble of the Soxhlet extractor and refluxed for 24 h with hexane, ethyl acetate and ethanol at a maximum temperature of 40°C. The liquid extract thus obtained was evaporated to dryness using a rotary evaporator at 40°C. The dried extracts of hexane, 12.68%, ethyl acetate, 7.46% and ethanol, 3.28% yield were prepared in a solution of olive oil for the pharmacological and toxicological tests.

Analgesic and anti-inflammatory activities of propolis sample

Experimental animals

Albino rats weighing between 70 and 120 g were obtained from the animal house of the Department of Chemical Sciences, University of Mkar, Mkar and used for the study. The experimental animals were kept in standard cages under standard conditions (25±2°C and a 12 h light/dark cycle) and maintained on standard pellets and drinking water *ad libitum* acclimatize for 5 days prior to commencement of the experiment. The animals were kept under hygienic conditions by constant cleaning and removal of faeces and spilled feeds from cages daily. The use and care of laboratory animals in the study were in accordance with ethical guidelines as contained in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (EEC Directive 86/609/EEC) of 1986.

Acute toxicity studies

The acute toxicity (median lethal dose LD_{50}) was determined using mice. Twenty five mice were grouped into five groups with five mice per group. The mice in each group were treated with propolis at doses of 100, 200, 300, 500 and 1000 mg/kg body weight .The experimental animals were observed for 72 h for signs and symptoms of toxicity (Arvouet-Grand et al., 1993).

Analgesic activity assay

Drug: Diclofenac sodium was used as a standard drug for the analgesic activity at a dose of 25 mg/kg body weight.

Acetic acid induced writhing test

Albino mice were induced with pain by intraperitoneal injection using 1% (v/v) acetic acid (2.3 mL/kg) as described by Santos et al. (1998). Animals were pre-treated with propolis sample prepared in olive oil at doses of 100, 200 and 300 mg/kg body weight orally, 45 min before acetic acid administration. Control animals received 2 mL volume of olive oil and the positive control animals were treated with the reference analgesic drug; diclofenac sodium (25 mg/kg). The number of abdominal constriction (writhings) was cumulatively counted over a period of 20 min after acetic acid administration. The percentage analgesic activity was calculated using the following formula (Santos et al., 1998).

$$\text{Analgesic activity (\%)} = \frac{\text{Mean writhing count (Control group-Treated group)}}{\text{Mean writhing count of control group}} \times 100$$

Anti-Inflammatory activity assay

Egg albumin induced oedema

The test was conducted using a modified method of Akah and Nwabie (1994). The rats were divided into five groups of three rats of either sex per group and were treated as follows: Group A which served as the negative control received olive oil (2 mL/kg bodyweight), while group B which served as the positive control received 25 mg/kg body weight of indomethacin, Groups C, D and

Table 1. Result of acute toxicity test on Propolis using hexane, ethyl acetate and ethanol solvents.

Dose (mg/kg)	Mortality		
	Hexane	Ethyl acetate	Ethanol
100	0/3	0/3	0/3
200	0/3	0/3	0/3
300	0/3	0/3	0/3
500	0/3	0/3	0/3
1000	0/3	0/3	0/3

(0/3); 0=number of deaths, 3=number of mice used per group for the assessment.
Source: Authors

E received 100, 200 and 300 mg/kg body weight of propolis orally, respectively. After 45 min of propolis sample administration, oedema was induced by sub-planter injection of 0.1 mL of fresh raw egg albumin in the left hind paw. Oedema volume was determined by measuring the paw volume using a thread. The readings were taken at 20 min intervals, that is, 0, 20, 40, 60, 80, 100, and 120 min after albumin administration.

The average increase in paw size of each group was calculated and compared with the control (olive oil) and indomethacin group.

Statistical analysis

The results were expressed as mean ± SEM. The statistical analysis was performed by means of Student's *t*-test, whereas Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test was used in order to compare more than two groups. $P \leq 0.05$ is indicative of significance.

RESULTS AND DISCUSSION

The use of propolis for the treatment of various ailments in which inflammation and pain are involved is cross-cultural and widespread. Its effect against diseases such as dermatological, odontological and gynaecological disorders has been widely reported (Castaldo and Capasso, 2002; De Castro, 2001). The mopping of free radicals generated by neutrophils during inflammation is the main mechanism of action for conventional anti-inflammatory drugs. It is also a well-known property of propolis (Paulino et al., 2003; El-Masry et al., 2011; Ichikawa et al., 2002). The process of inflammation includes production and release of mediators from neurons or damaged tissues which lead to various responses including pain (Paulino et al., 2003).

Hexane, ethyl acetate and ethanol extracts of propolis is non-toxic at 1000 mg/kg bodyweight (Table 1). This is in line with several reports of the relatively low toxicity of propolis. Arvouet-Grand et al. (1993) reported the oral LD_{50} of propolis extract to be greater than 7340 mg/kg in mice (Abozid and Ahmed, 2013). Dobrowolski et al. (1991) administered approximately 700 mg/kg orally to groups of 10 mice (five males and five females) and monitored them for 48 h. No deaths were recorded and the preparations were well tolerated by the test animals. In another study, Havsteen (1983) reports LD_{50} of flavonoids to range from 8000 to 40000 mg/kg bodyweight of rats. Considering the main component of propolis to be flavonoids, it should be relatively safe since flavonoids are of very low toxicity.

Anti-inflammatory activity

Figures 1, 2, 3, 4, 5 and 6 indicate that Propolis sample at 300 mg/kg bodyweight showed a steady increase in inhibition from 20 to 100 min. This result is better than the standard drug, indomethacin. The inhibitory effect of propolis at 200 mg/kg weight administration is also better than indomethacin. The best result was observed with 200 mg/kg bodyweight administration of sample. The inhibitory effect observed on the n-hexane extract of propolis is in a dose dependent manner. The higher the dosage, the more the inhibition. Inflammation is presumably initiated by the release of histamine, kinin, fibrinolysin and phospholipase A_2 (Parandin and Daroogari, 2019). These intermediaries of inflammation induce oedema by vasodilation and increased vascular permeability (Zhang et al., 2015; Li et al., 2011; Xu et al., 2014; Uwaya et al., 2020). The anti-inflammatory effect of this sample may be due to the decrease in the amount of the induced intermediaries earlier mentioned.

The inhibition of inflammation by ethyl acetate is not dose dependent. No regular pattern is observed with its administration. However, better anti-inflammatory activity is observed at 200 mg/kg bodyweight administration at 60 minutes as seen in figure 4. This too is better than the standard drug, indomethacin.

Figure 6 shows better inhibitory activity of ethanol fraction of propolis at 300 mg/kg bodyweight administration. The activity is time dependent. In the earlier phase, its activity at 300 mg/kg bodyweight administration is not as good as indomethacin. In the later phase; 60-120 min (Figures 4, 5, 6 and 7), the inhibition of inflammation is more pronounced and gets better than indomethacin.

Major constituents of propolis apart from resins and waxes include flavonoids, terpenoids and phenolic acids

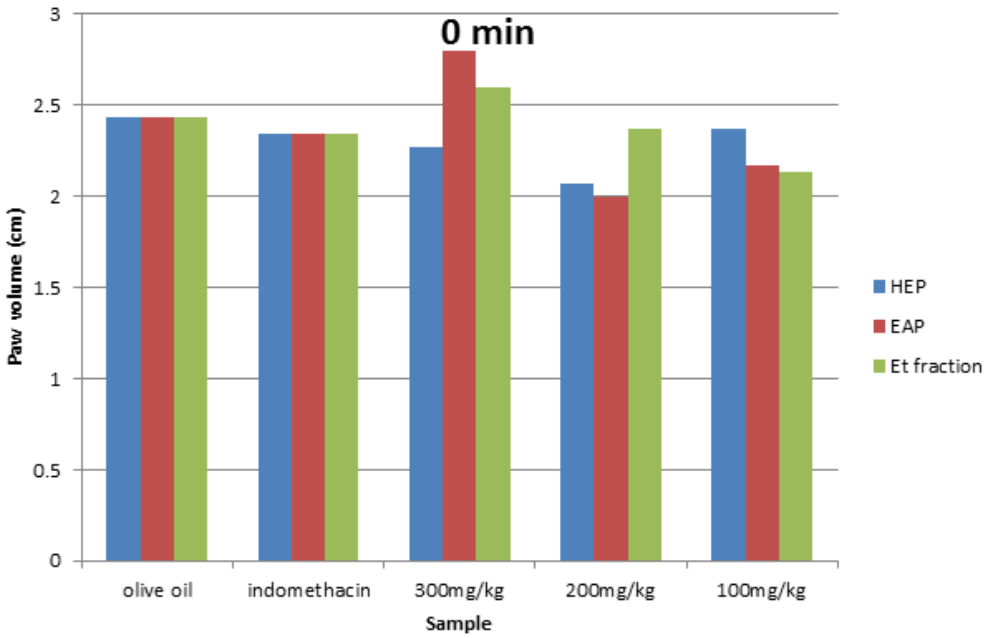

Figure 1. Comparative anti-inflammatory activities of hexane (HEP), ethyl acetate (EAP) and ethanol (Et) extracts of propolis at 0 min.
Source: Authors

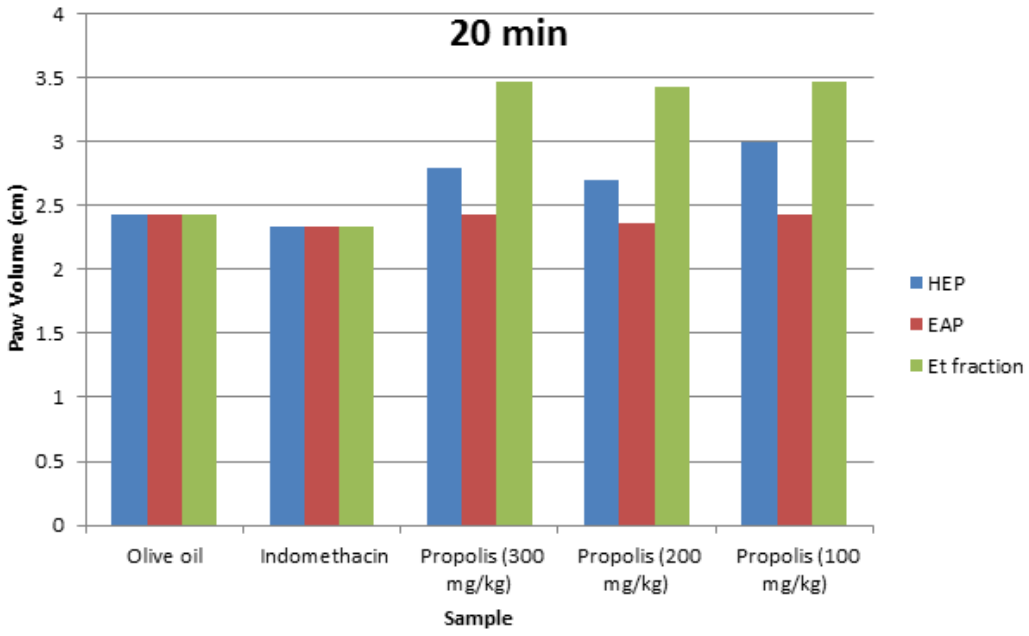

Figure 2. Comparative Anti-inflammatory activities of hexane (HEP), ethyl acetate (EAP) and ethanol (Et) extracts of propolis at 20 min.
Source: Authors

such as cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid and their derivatives (Razmi et al., 2013; Pimenta et al., 2015; Sforcin, 2016). Parandin and Daroogari (2019) reported flavones, flavonols, flavanones, flavanonols, chalcones, dihydrochalcones, isoflavones, isodihydroflavones, flavans, isoflavones and

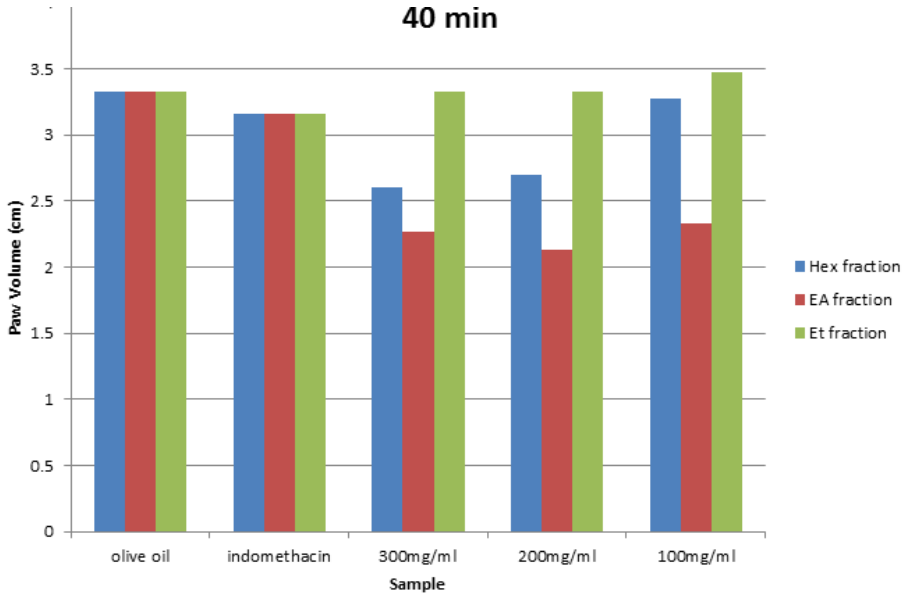

Figure 3. Comparative anti-inflammatory activities of hexane (HEP), ethyl acetate (EAP) and ethanol (Et) extracts of propolis at 40 min.
Source: Authors

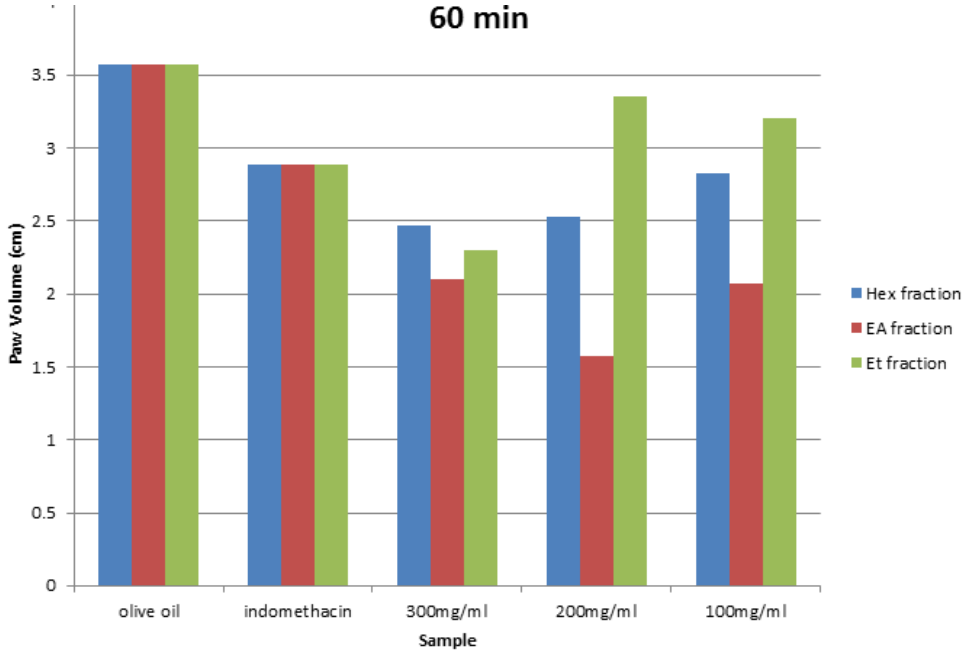

Figure 4. Comparative anti-inflammatory activities of hexane (HEP), ethyl acetate (EAP) and ethanol (Et) extracts of propolis at 60 min.
Source: Authors

neoflavonoids as types of flavonoids found in propolis. Several studies show that phytochemicals including flavonoids, phenolic acids and terpenoids have antimicrobial (Inui et al., 2014; Brodowska, 2017; Górniak et al., 2019), antinociceptive, anti-inflammatory and antioxidant properties (Murugan and Parimelazhagan, 2013; Wang et al., 2014; Yamanishi et al., 2014; Fazio et al., 2013; Parandin and Daroogari, 2019; Fernandez et

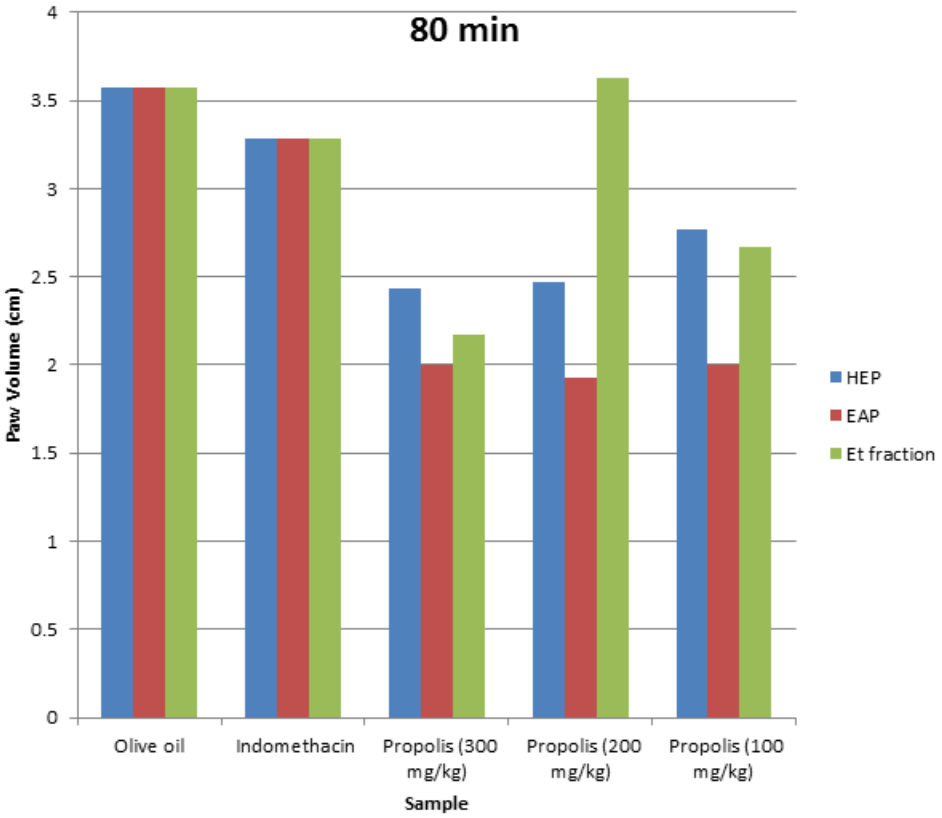

Figure 5. Comparative anti-inflammatory activities of hexane (HEP), ethyl acetate (EAP) and ethanol (Et) extracts of propolis at 80 min.
Source: Authors

al., 1998; Yonathan et al., 2006; Xu et al., 2016; Zhao et al., 2016). This is because they have been reported to prevent the production of inflammation intermediaries such as prostaglandins, arachidonic acid, histamine, bradykinins (Amresh et al., 2007; Mirazi and Hosseini, 2013), and cyclooxygenase 2 (Razmi et al., 2013) which are involved in inflammation and pain.

The solvent used during extraction determines the biological activity obtained (Devequi-Nunes et al., 2018). Similar biological activity may be more pronounced with increased polarity as concluded by Wieczynska et al. (2017), who reported a stronger antimicrobial activity of ethanolic extracts than hexane extracts of polished propolis. This improved anti-inflammatory activity of ethanolic extract of propolis obtained from Gboko may be as a result of increased availability of flavonoids and other phenolics due to the high polarity of ethanol.

Analgesic activity

Hexane is highly selective during extraction. Figure 8 shows that hexane extract at 300 and 100 mg/kg bodyweight administration gave an analgesic activity of 78.14%. This result appears better than that of the standard analgesic used (diclofenac sodium). Best analgesic activity was recorded at 200 mg/kg bodyweight dosage which was 100% (Figure 8). This is not dose dependent.

Paulino et al. (2003) reported that the main constituents of propolis in temperate zones are flavonoids, whereas in the tropics other classes such as aromatic derivatives, specific terpenoids and prenylated p-coumaric acids and acetophenones may be found. In spite of these many variations in their chemical composition, they have several biological activities in common (Bankova et al., 1995; Seidel et al., 2008).

Bonvehi et al. (1994) reported poor result when determination and prediction of biological activity of propolis was based on analysis of individual compounds. This further proves the assertion that, biological activity of propolis is based on synergistic activity of individual compounds (Boisard et al., 2015; Chen and Shen, 2008).

Less activity was observed in ethyl acetate fraction. Figure 8 shows the standard drug, diclofenac had better activity than ethyl acetate fraction at 300 and 100 mg/kg dosages. The best analgesic activity was recorded at 200 mg/kg administration as seen in figure 8. The analgesic effect of propolis obtained from Gboko against acetic acid induced writhing may be due to local peritoneal receptors

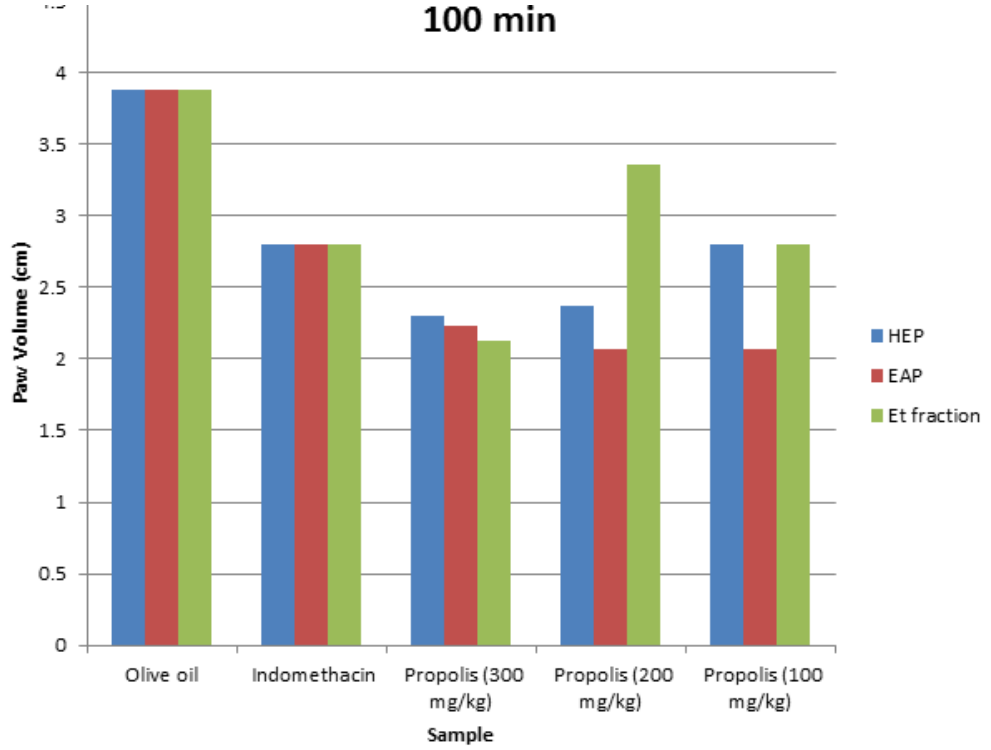

Figure 6. Comparative anti-inflammatory activities of hexane (HEP), ethyl acetate (EAP) and ethanol (Et) extracts of propolis at 100 min.
Source: Authors

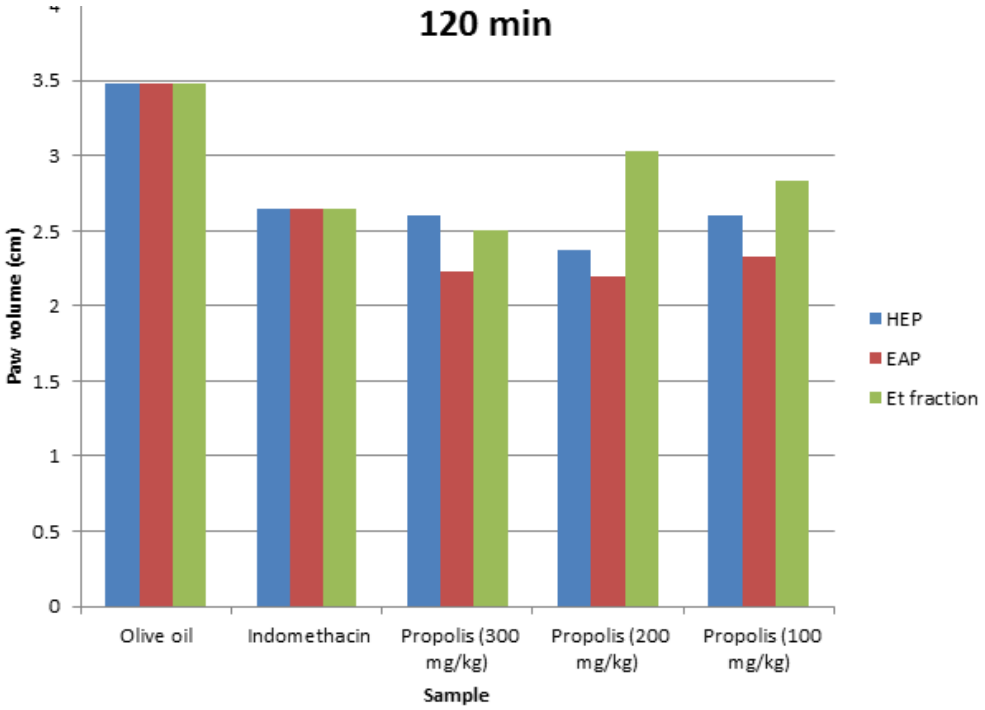

Figure 7. Comparative anti-inflammatory activities of hexane (HEP), ethyl acetate (EAP) and ethanol (Et) extracts of propolis at 120 min.
Source: Authors

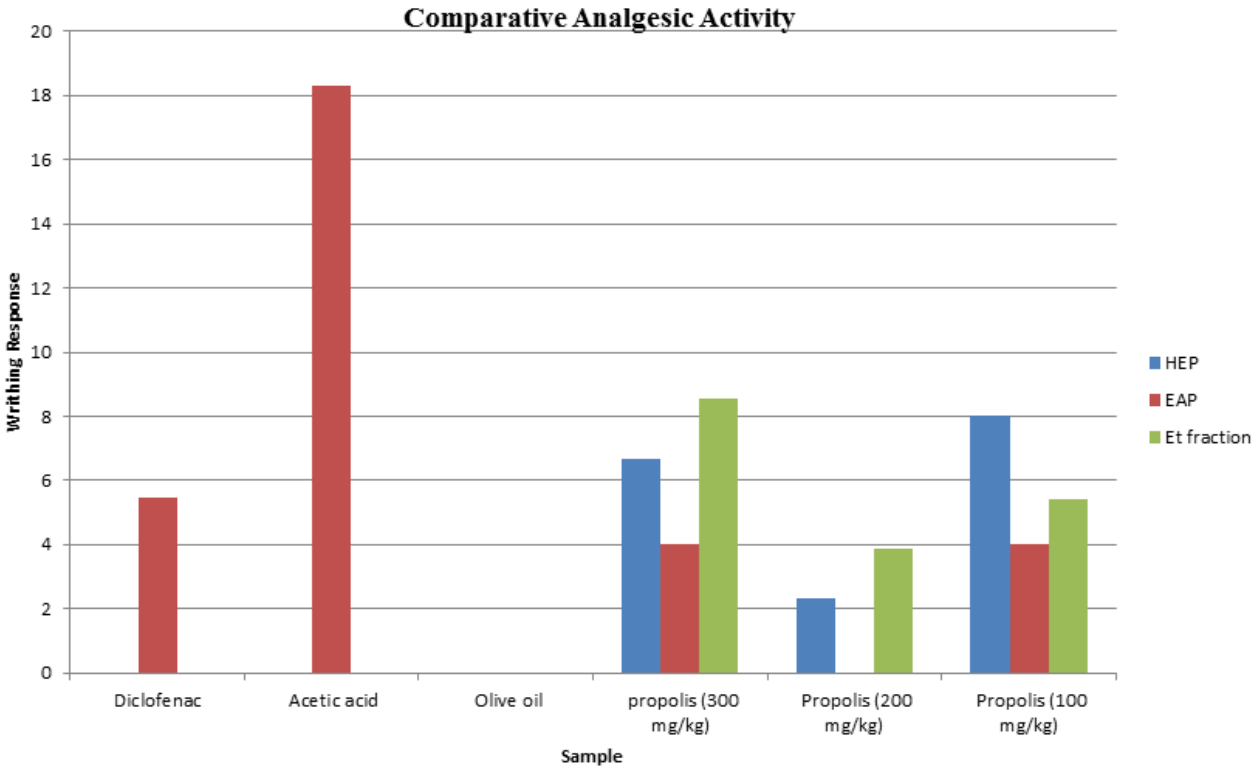

Figure 8. Comparative analgesic activities of hexane (HEP), ethyl acetate (EAP) and ethanol (Et) extracts of propolis.
Source: Authors

or by inhibition of prostaglandin synthesis or activity (Adzu et al., 2001; Atta and Alkofahi, 1998).

Figure 8 shows that at 300 mg/kg dosage, ethanol fraction of propolis gave a relatively poor analgesic activity compared to 200 and 100 mg/kg dosage and diclofenac sodium. The component of propolis extracted, are more polar at this stage considering the high polarity of ethanol.

According to Ristivojević et al. (2015), non polar fractions of propolis contain mostly waxes and hydrocarbons which include alkanes, alkenes, alkadienes, monoesters, diesters, aromatic esters, fatty acids and steroids which in most cases do not exhibit any significant pharmacological activity. The observed activity of the n-hexane fraction may be due to a synergistic activity of the non polar components of the propolis. This is in line with the report of Ristivojević et al. (2015) that no specific compound can be associated with the pharmacologic properties of propolis.

Boisard et al. (2015) also reported that different combinations of phenolic compounds involving a complex mechanism of action are essential for biological activity of propolis. For poplar type of propolis, biological activity can be attributed to the synergistic effect of phenolic compounds including cinnamic acid and ester derivatives, including caffeic acid and Caffeic Acid PhenethylEster (CAPE), and flavonoids including pinocembrin, pinobanksin, galangin, chrysin and naringenin (Bankova, 2005; Vardar-Ünlü et al., 2008).

This activity of ethanol fraction of propolis from Nigeria agrees with ethanol extract of propolis from Bulgaria (Paulino et al., 2003), Iran (Parandin and Daroogari, 2019), and China (Sun et al., 2018). It is also in agreement with the result of hydroalcoholic propolis of Brazilian origin (Cavendish et al., 2015), water of Iraqi origin (Abdel Mahdi Kassim Altaee, 2014) and Moroccan origin (Mountassir et al., 2014) which have shown good anti-inflammatory and antinociceptive activities in animal models as reported by Al-Hariri and Abualait (2020).

The findings on analgesic study on propolis are in agreement with studies by Sun et al. (2018) which reported that different fractions from Chinese propolis extracts enriched in polyphenolic constitutions showed central and peripheral antinociceptive effects in animal models. It is also in line with the report of Cavendish et al. (2015) which concluded that the hydroalcoholic extract of Brazillian red propolis exhibited anti-inflammatory and neurogenic pain inhibition without emotional and motor side effects in rodents.

Conclusion

The varying polarity of solvents used, has proved that

pharmacological activities of sample fractions of propolis is as a result of synergism rather than single fraction action. This can be seen in the good analgesic activity of hexane, a non-polar solvent and ethanol, a highly polar solvent as reported. The results show that this propolis sample is non-toxic at very high doses. It also shows that both non-polar and polar extracts of propolis obtained from Gboko, Benue State have anti-inflammatory and analgesic activities at various dosages.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of different concentrations of plant growth hormones on callus induction and regeneration of Shea tree (*Vitellaria paradoxa*)

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The slow natural regeneration pattern of the Shea tree due to the long gestation period and intense harvest has limited the domestication and genetic improvement of this tree. These limitations have necessitated the need for an alternative method of conserving the Shea tree outside the natural habitat. The propagation of the Shea tree by the *in-vitro* clonal technique presents such an alternative method. The purpose of this study was to determine the optimal concentration of 2, 4-dichlorophenoxyacetic acid (2, 4-D) and Picloram in Murashige and Skoog medium for callus formation and regeneration. The first experiment was done to achieve the best surface sterilization method and the effect of different concentrations of 2, 4-D or Picloram on callus formation. Callus induction percentage (CI%) of the explants in Murashige and Skoog medium were evaluated. The basal media were supplemented with 30 g/L of sucrose, 2.8 g/L phytagel and combinations of 2, 4-D or Picloram in various concentrations (1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 mg/L) replicated four times with five explants in each bottle. From the result, the leaf explants soaked in 70% ethanol for 1 min and 1% sodium hypochlorite for 15 min with 1 µl of tween 20 had the highest percentage (100%) of sterile leaf explants and showed no contaminations both in the leaf and media. Callus was induced at 2 weeks of culturing in all the treatments except for MS basal without growth hormone which induced no callus. A concentration of 1.5 mg/ L 2, 4-D gave the best callus. The highest CI% (100%) was shown at 4 weeks in MS + 3.5 mg/L picloram media. The callus was light in colour and friable in texture. The result indicated that Picloram gave better callus compared to other treatments and will give a better response for regeneration of Shea tree.

Key words: Explant, callus induction, 2, 4-d, picloram, shea tree and Regeneration.

INTRODUCTION

Shea tree (*Vitellaria paradoxa*) belongs to the family Sapotaceae. It grows widely in the savannah region of West African Countries (Maranz and Wiesman, 2003). It is a deciduous dicotyledonous crop that has a gestation period varying from 15 to 20 years. The Shea tree is of great economic importance due to its multipurpose use (Yakubu et al., 2015). It serves as a fruit tree as well as an oilseed crop. It is popular for the Shea butter produced from it, which is relevant in the food, cosmetic and pharmaceutical industries (Boffa, 2015). Plant hormones are chemicals that regulate plant growth. They are signal molecules produced at a specific location in the plant and

extremely low concentrations. Hormones are naturally produced within plants, though very similar chemicals are produced by fungi and bacteria that can affect plant growth (Srivastava, 2002). The most widely used plant growth hormones are auxins and cytokinins. The types, concentrations and the ratio of these growth regulators used for media supplementation are essential for the type of culture responses obtained (Anca, 2009). Naturally, the Shea tree grows and regenerates itself in the wild but its slow and poor natural regeneration pattern due to long gestation period; impacts of bush fires and desertification have limited the domestication and genetic improvement of this crop. These limitations have necessitated the need for an alternative method of conserving this plant's genetic resource outside the natural habitat. The propagation of the Shea tree by the *in-vitro* clonal technique presents such an alternative method (Lovett and Haq, 2013). However, vegetative methods, such as grafting, budding, cuttings and air-layering, have only produced limited success (Yeboah et al., 2010). The micropropagation of woody tree species is now a method widely used in the regeneration and conservation of germplasms (Lovett and Haq, 2013). Callus induction and plant regeneration are some of the key tools in plant biotechnology that exploits the totipotent nature of plant cells (Mukherjee et al., 2011).

Tissue culture serves as an indispensable tool for transgenic plant production. For nearly any transformation system, an efficient regeneration protocol is imperative (Cardoza, 2008). Due to the lack of proper cultivation practices, destruction of plant habitats, excessive and indiscriminate collection of medicinal plants for the supplement of global demands on herbal medicine, many medicinal plants like *Salacia chinensis* and *Vitelleria paradoxa* are severely threatened. Therefore, to conserve and rapidly propagate the rare and endangered medicinal plants, advanced biotechnological methods of culturing plant cells and tissues like micro propagation methods are employed (Nalawade et al., 2001). This study aims to evaluate the effect of different concentrations of plant growth hormones on callus induction and regeneration of Shea trees. Also, to produce callus from explant of the Shea tree and determine the optimal concentration of 2, 4-D and Picloram media for callus formation and plantlet regeneration.

MATERIALS AND METHODS

Study location

The study was carried out in Agricultural Biotechnology Laboratory at Science and Technology Complex (SHETSCO), Sheda, located at the outskirt of Abuja, North Central Nigeria with Latitude 8˚51'25' N and Longitude 7˚02'39''E.

Plant material used for callus induction

A three days old tender leaf of Shea butter was used as an explant for the callus induction.

Treatment and experimental design

The treatments consist of six different concentrations of 2, 4 –D and Picloram (4.5, 4, 3.5, 3, 2.5, and 1.5 mg/L) arranged in a completely randomised design (CRD) with four replications.

Explant preparation and surface sterilization.

The three days old leave explants were plucked off from the mother plant using a sterile knife and then taken to the laboratory. The explants were washed vigorously under running tap water with drops of liquid detergent to remove the dirt particles then followed by rinsing with distilled water. The laminar flow hood was sprinkled with 70% Ethanol to control contamination and the burner was lit. To surface sterilize the tender leaves used as explants, ethanol and sodium hypochlorite method of surface sterilization were adopted. However, due to lack of standing protocol for surface sterilization of Shea butter explants for callus induction, three different ethanol and sodium hypochlorite combinations were used with varying concentrations and timing as follows:

S0: Explants were only washed with distilled water 5-6 times.
S1: Explants soaked in 70% ethanol for 1 min then soaked in 1% sodium hypochlorite for 15 min with 1 µl of tween 20.
S2: Explants were soaked in 70% ethanol for 2 min then soaked in 2% sodium hypochlorite for 20 min with 1µl of tween 20.
S3: Explants were soaked in 70% ethanol for 3 min then soaked in 3% sodium hypochlorite for 10 min with 1µl of tween 20.

Using the surface sterilization methods above, the explants were soaked in 70% ethanol first at S1, S2 and S3, then rinsed three times with sterile water. Secondly, the explants were soaked in sodium hypochlorite with 1 µl tween 20 then rinsed four times with sterile water. After surface sterilization, all explants were trimmed to small sizes of 2 mm by 2 mm and then cultured on MS medium without growth hormone to check out for contaminations and sterility within four days. Percentage sterility was recorded.

Preparation of MS media and callus induction

Two different plant growth regulators (PGRs; 2, 4-D and Picloram) was used for callus induction in shea butter. Six different concentrations of each auxin (4.5, 4, 3.5, 3, 2.5, and 1.5 mg/L) were used in Murashige and Skoog media (Murashige and Skoog 1962) as a basal medium and a control treatment with no growth hormone was included (Figure 2). Murashige and Skoog culture medium (MS) was supplemented with 30 g/L sucrose, as a support material (Khan et al., 2001) and 100 g/l ascorbic acid. The pH was adjusted

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to 5.7 using 1 M of NaOH. Pyhtagel was added as gelling age the medium at a concentration of 2.8 g/L before the medium was sterilized at psi 121°C for 15 min. At the end of the sterilization, the media was allowed to cool. Different concentrations of the auxins (2, 4-D or Picloram) was added using a microfilter and the medium was dispensed into the bottle in the laminar flow hood and allowed to settle for 24 h. Sterile explants were cultured into the bottle and placed about 2 cm apart on the culture medium. Twenty explants were used in all the media with each bottle having five explants and four bottles per media. The bottle was sealed in a polythene bag and stored in the dark growth room 28°C. The two sets of the experiment (MS + 2, 4-D and MS + Picloram) was conducted concurrently. The performance of the culture and callus produced was evaluated visually at 2 and 4 weeks and recorded. The cultures were monitored, every four weeks for sub-culturing and development noted. After four weeks, the explants were separated from the callus formed and the callus was moved into a fresh media for regeneration. The criteria for scoring explants for callus is based on the number of explants producing callus per media according to Amoo and Ayisire (2005).

Data collections

1. Percentage sterility was calculated using the formula:

$$\text{Percentage sterility} = \frac{\text{Number of Uncontaminated explants}}{\text{Total number of cultured explants}} \times 100$$

(Nwala, 2012).

2. Callus Induction Percentage: Percentage of callus induction was calculated using the formula:

$$\text{Callus Induction Percentage (\%)} = \frac{\text{Number of explants with callus}}{\text{Total number of explants inoculated}} \times 100$$ (Kabir et al., 2008).

3. Degree of callus formed was rated using the scale; **+** = Very Poor, **+ +** = poor, **+ + +** = Good, **+ + + +** = Very good (Dhiya et al., 2013).

Statistical analysis

Data collected were subjected to analysis of variance (ANOVA) using statistical analysis software (SAS). Means were separated using Duncan's multiple range test (DMRT) at a 5% level of probability.

RESULTS

Effect of different sterilant combinations on leaf explants of *Vitelleria paradoxa* for tissue culture establishment

Table 1 summarizes the efficiency of the different combinations of ethanol and sodium hypochlorite at different times and concentrations. From the result, it was observed that the method of soaking the leaf explant in 70% ethanol for 3 min and then 3% sodium hypochlorite for 10 min had the lowest percentage (15%) of sterile leaves. Out of the 20 explants, only 3 explants were sterile while others were contaminated and burnt. The soaking of explants in a combination of 70% ethanol for 2 min and 2% of sodium hypochlorite for 20 min showed a higher percentage of 75% of sterile leaves. Leaf explants soaked in a combination of 70% ethanol for 1 min and 1% sodium hypochlorite for 15 min had the highest percentage (100%) of sterile leaf explants and shows no contaminations both in the leaf and media. The leaves remained green and sterile for ten days. Also, washing the leaf explants with only distilled water had no sterile leaf explants (Figure 1).

Effect of MS media supplemented with different concentrations of 2, 4 –D for callus formation on leaf explants at 2 weeks and 4 weeks interval.

From the observations, at 2 weeks all the media had at least one explant induced callus except for the MS basal (control) which had no growth regulator. At 2 and 4 weeks, media MA1 and MB1 had the least callus induction percentage of 5% and 10%, respectively. At 2 weeks, MD1 and ME1 had 20% same as the media MC1 and MD1 at 4 weeks; although, media ME1 performed better with percentage value of 30% at 4 weeks. However, MF1 performed better with (55%) than other treatments at 2 weeks while at 4 weeks, MF1 had the highest percentage value of 75% of callus formed. Tables 2 and 3 show the analysis of variance using 2, 4 –D on leaf explant of the Shea tree. There were significant differences among treatments at 2 weeks and 4 weeks of culturing. However, there was no significant (p> 0.05) difference at 2 weeks and 4 weeks in media MA1, MB1, MC1 and MD1. Media MA1 and MB1 had the lowest mean value of 0.25 at 2 weeks and 0.5 at 4 weeks. At 2 weeks. The differences between media MD1 and ME1 was not significant (p>0.05). Also, media MF1 had the highest mean value of 2.75 and 3.75 and was significantly (p> 0.05) different from others in 2 and 4 weeks. Media MA1 and MB1 at 2 and 4 weeks produced very poor calli while MC1, MD1 and ME1 produced poor calli. However, media MF1 had good calli at 2 weeks and then produce very good calli at 4 weeks. Only MS basal produced no callus at 2 weeks and 4 weeks. The colour and morphology of calli produced in media MA1, MB1, MC1, MD1 and ME1 were compact and whitish except for media MF1 which calli were friable and whitish.

Effect of MS media supplemented with different concentrations of picloram on callus formation from leaf explants of Shea tree at 2 and 4 weeks intervals

Tables 4 and 5, show a positive response of explants to the different concentrations of picloram at 2 weeks of culturing. Media MA2, ME2 and MF2 at 2 weeks had the least callus percentage induction value of 20% while at

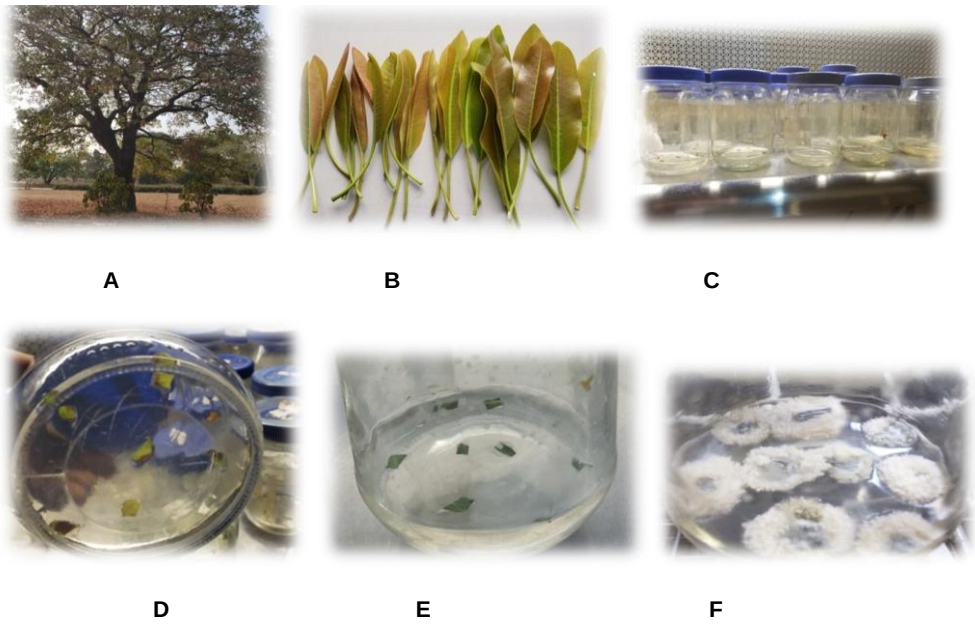

Figure 1. A- Source of explant (Mother plant), **B**- Leaf explants, **C-** Surface sterilized explants, **D-** Sterile explants, **E**- Sterile leaf explant, **F**- Contaminated explant.
Source: Authors

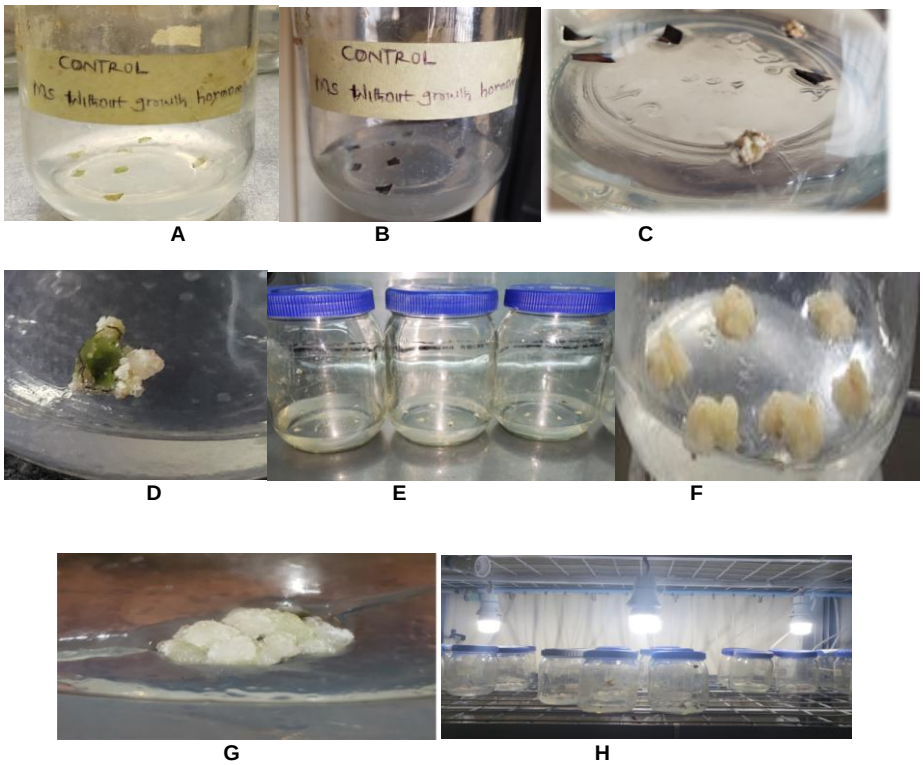

Figure 2. A- Explant in MS basal after culture, **B**- Explant in MS basal after 4 weeks, **C**- Callus from 4.5 mg/ L 2, 4 D, **D**- Callus from 3.5 mg/L Picloram, **E**- Callus subcultured for Multiplications, **F, G**- Callus multiplied in 3.0 mg/L Picloram after 4 weeks; **H**- The experiment in the growth culture room under light
Source: Authors

Table 1. Effects of different sterilant combinations on leaf explants of *Vitelleria paradoxa* for callus induction establishment.

Sterilant concentration/Time (mins)	No of healthy sterile leaves	% sterility of explants
S0 - 20 leaves soaked in distilled water and rinse 4 to 6 times	0	0
S1 - 20 leaves soaked in 70% ethanol for 1 min + 1% sodium hypochlorite for 15 min + 1 µl of tween 20	20	100
S2 - 20 leaves soaked in 70% ethanol for 2 min + 2% sodium hypochlorite for 20 min + 1 µl of tween 20	15	75
S3 - 20 leaves soaked in 70% ethanol for 3 min + 3% sodium hypochlorite for 10 min + 1 µl of tween 20	3	15

Source: Authors

Table 2. Effect of MS media supplemented with different concentrations of 2, 4-D on callus formation from leaf explants of Shea tree at 2 weeks.

Growth regulators 2,4-D	Callus induction percentage (%)	(Mean ± SE)	Degree of callus	Colour and morphology
Control	0	0	-	NC
MA1	5	0.25^{bc} ± 0.25	+	CW
MB1	5	0.25^{bc} ± 0.25	+	CW
MC1	15	0.75^{bc} ±0.25	+	CW
MD1	20	1.00^{b}±0.41	+ +	CW
ME1	20	1.00^{b}±0.41	+ +	CW
MF1	55	2.75^{a}±0.25	+ + +	FW

Mean with the same letters are not significantly different according to Duncan's multiple range test (DMRT) at p= 0.05. MA1 - MS + 4.5mg/L 2, 4-D, MB1- MS+ 4.0mg/L 2, 4-D, MC1- MS+ 3.5mg/L 2, 4-D, MD1- MS+ 3.0mg/L 2, 4-D, ME1- MS + 2.5mg/L 2, 4-D, MF1- MS+ 1.5mg/L 2, 4-D, FW- Friable White, CW- Compact White, - No Callus, + Very poor callus, ++ Poor callus, +++ Good callus, ++++ Very good callus.
Source: Authors

4 weeks media MA2, ME2 and MF2 had percentage value of 35%. Likewise, MB2 at 2 weeks but at 4 weeks, there was an increase in callus formed in ME2. However, at 2 weeks and 4 weeks MD2 performed better than all other treatments but media MC2 had the best result at 2 weeks and 4 weeks. At 4 weeks, media MC2 had the highest percentage value of 100% which gave the highest calli formed. In the mean value, there were significant (p<0.05) differences among the treatment. However, there were no differences at 2 weeks and 4 weeks in the media MA2, ME2 and MF2 respectively. At 2 weeks, media MA2, ME2 and MF2 gave the least mean value of 1.0 while at 4 weeks, media MA2, MF2 had the least mean value of 1.75; likewise media MB2 at 2 weeks. Also, at 2 weeks MC2 had the highest mean value of 3.75 and was significantly (p<0.05) different from others while at 4 weeks MC2 gave the highest mean value of 5.0 which is the best and significantly (p<0.05) different from others. From the observations, the degree of callus produced at 2 weeks from media MA2, MB2, ME2 and MF2 was poor likewise media MA2 and MF2 at 4 weeks. At 2 weeks, media MD2 produced good calli likewise media MB2 and MC2 at 4 weeks. Therefore, media MC2 and MD2 produced the best calli and was used for the regeneration of Shea plantlet. All the treatments MA2, MB2, MC2, MD2, ME2 and MF2 produced friable and whitish calli except for control which had no callus.

DISCUSSION

The first experiment was to investigate the suitable surface sterilization method for leaf explants of the Shea tree. It was observed that soaking the leaf explants in 70% ethanol for 1 min and 1% sodium hypochlorite for 15 mins with a drop of tween 20 gave the highest percentage (100%) of sterile leaves. This was also observed by (Ghosh, 2005), In the case of *Salacia chinensis* the surface sterilization with 70% ethanol for 1 min followed by sodium hypochlorite (1% + 1µl of Tween 20) for 15 min proved most effective for maximum survival percentage in leaf explants. Soaking the explants in 70% ethanol for 3 mins and 3% sodium hypochlorite for 10 mins had the lowest percentage (15%) of sterile leaf establishment because of the increased time in ethanol and increased concentration of sodium hypochlorite. It was also reported by Sharma et al. (2014) that increasing the time of exposure to sodium hypochlorite significantly reduced contamination but showed an adverse effect on explants. Also, when the

Table 3. Effect of MS media supplemented with different concentrations of 2, 4-D on callus formation from leaf explants of Shea tree at 4 weeks.

Growth regulators 2,4-D	Callus induction percentage (%)	(Mean ± SE)	Degree of callus	Colour and morphology
Control	0	0	-	NC
MA1	10	0.50^{cd}±0.29	+	CW
MB1	10	0.50^{cd} ±0.29	+	CW
MC1	20	1.00^{bc}±0.41	+ +	CW
MD1	20	1.00^{bc}±0.41	+ +	CW
ME1	30	1.50^{b}±0.29	+ +	CW
MF1	75	3.75^{a}±0.25	+ + + +	FW

Mean with same letters are not significantly different according to DMRT at p =0.05.
MA1- MS + 4.5mg/L 2, 4-D, MB1- MS+ 4.0mg/L 2, 4-D, MC1- MS+ 3.5mg/L 2, 4-D, MD1- MS+ 3.0mg/L 2, 4-D, ME1- MS + 2.5mg/L 2, 4-D, MF1- MS+ 1.5mg/L 2, 4-D, FW- Friable White CW- Compact White, - No Callus, + Very poor callus, ++ Poor callus, +++ Good callus, ++++ Very good callus.
Source: Authors

Table 4. Effect of MS media supplemented with different concentrations of picloram on callus formation from leaf explants of Shea tree at 2 weeks.

Growth regulators picloram	Callus induction percentage (%)	(Mean ± SE)	Degree of callus	Colour and morphology
Control	0	0	-	NC
MA2	20	1.00^{d}±0.0	+ +	FW
MB2	35	1.75^{c} ± 0.25	+ +	FW
MC2	75	3.75^{a}±0.25	+ + + +	FW
MD2	45	2.25^{b}±0.25	+ + +	FW
ME2	20	1.00^{d}±0.0	+ +	FW
MF2	20	1.00^{d}±0.0	+ +	FW

Mean with the same letters are not significantly different according to DMRT at *p* =0.05.
MA1- MS + 4.5mg/L Picloram, MB1- MS+ 4.0mg/L Picloram, MC1- MS+ 3.5mg/L Picloram, MD1- MS+ 3.0mg/L Picloram, ME1- MS + 2.5mg/L Picloram, MF1- MS+ 1.5mg/L Picloram, FW- Friable White, CW- Compact White, - No Callus, + Very poor callus, ++ Poor callus, +++ Good callus, ++++ Very good callus.
Source: Authors

leaves were washed with distilled water only without tween 20, it had no sterile leaf all the leaves were contaminated. This was also confirmed by Thomas and Puthur (2002) that in an experiment where tween 20 was deliberately omitted there was an increase in the percentage of contamination. Tween 20 is said to be a surfactant that act as a detergent which attaches itself to possible oil exudates helping to establish clean explants. The result of the present study on the effect of 2, 4 –D or Picloram on callus formation of Shea tree shows that basal media without plant growth regulator did not induce any callus growth and this was also observed by Dhiya et al. (2013) and Namrata et al. (2014) in the study on the influence of hormones and explants towards in vitro callusing and shoot organogenesis in a commercially important medicinal plant, callus was also not induced in media without growth regulators. The effect of different growth regulators on callus induction and on callus growth was the objective of this study. It was observed that to induce callus 2, 4-D medium was not effective enough whereas the callus remains healthy and increase in different concentration of picloram combination. The best callus initiation was observed with 2, 4-D in 1.5 mg/L, it showed friable callus. Thus the whole outcome showed that 2, 4 -D was not efficient for callus induction because of its toxic nature and also confirmed it that 2, 4-D is used as a form of herbicide to kill weeds. The result showed that when the concentration is high it kills the leaves.

The current result recorded that at 4.5 mg/L 2, 4-D and 4.5 mg/L picloram the callus induction percentage was low because of the increase in the concentration of the auxins. However, high concentrations of the Auxins are toxic to the plant cells. These facts explain the increase in the callus fresh weight as the concentration of the Auxins increased up to a specific level. High concentration of 2, 4-D (4. 5 mg/L) gave fewer calli than 1.5 mg/ L. The callus was watery and non regeneratible type. This might be due to Ethylene production which reduced cell division. Synthetic auxin like 2, 4-D has been developed as herbicide to control weed. The presence of growth regulators in culture medium is an important factor in

Table 5. Effect of MS media supplemented with different concentrations of picloram on callus formation from leaf explants of Shea tree at 4 weeks.

Growth regulators picloram	Callus induction percentage (%)	(Mean ± SE)	Degree of callus	Colour and morphology
Control	0	0	-	NC
MA2	35	1.75^{d}±0.25	+ +	FW
MB2	50	2.50^{c} ± 0.29	+ + +	FW
MC2	100	5.00^{a}±0.0	+ + + +	FW
MD2	80	4.00^{b}±0.41	+ + + +	FW
ME2	40	2.00^{cd}±0.0	+ + +	FW
MF2	35	1.75^{d}±0.25	+ +	FW

Mean with the same letters are not significantly different according to DMRT at p =0.05. MA1- MS + 4.5 mg/L Picloram, MB1- MS+ 4.0mg/L Picloram, MC1- MS+ 3.5 mg/L Picloram, MD1- MS+ 3.0 mg/L Picloram, ME1- MS + 2.5 mg/L Picloram, MF1- MS+ 1.5 mg/L Picloram, FW- Friable White, CW- Compact White, - No Callus, + Very poor callus, ++ Poor callus, +++ Good callus, ++++ Very good callus.
Source: Authors

callus proliferation, growth and differentiation of cultured cells (Kalidass et al., 2010). Liu et al. (2006) reported that 2, 4-D had been used singly and in combination with cytokinins to enhance callus induction and maintenance. In the present study, callus induction was observed after two weeks of inoculation. Adu-Gyamfi et al. (2012) also observed callus after the same duration though the explant used was cotyledon. Callus was initiated at the cut ends of the leaf explant of Shea. Callus formation at the proximal end of the explant in this study was in line with reports on *Pegannum harmla* (Saini and Jaiwal. 2002). The result of the present study on effect of picloram on callus induction 3.5 mg/L gave the best CI% of (100%) the callus is friable and creamy in appearance. According to Trigiano and Gray (2005) rapid growth and light colour callus usually indicate a healthy culture and a friable callus is a very suitable for breaking up either for sub culturing or to produce plantlets.

Conclusion

In this study, the best sterilization method of shea leaf explant was by soaking in 70% ethanol for 1 min followed by 15 min in 1% sodium hypochlorite with 1 µl of Tween 20. The optimum concentration for callus induction was 3.5 mg/L Picloram in MS media. *In-vitro* propagation of shea tree could serve as a viable alternative for raising woody plants with propagation difficulties. Application of appropriate hormones in the right proportion could also enhance tree growth response.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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Influence of arbuscular mycorrhizal fungi and phosphorous on the growth, nutrient uptake, chlorophyll content and some metabolites of eggplant (*Solanum melongena* L. VAR. Yalo) under saline conditions

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The effects of arbuscular mycorrhizal fungi (AMF) and phosphorous (P) on the growth, nutrient uptake, chlorophyll content and some metabolites of eggplant (*Solanum melongena* L. VAR. Yalo) were determined under saline conditions, through a greenhouse experiment conducted for six weeks. Seedlings were planted in polythene bags previously filled with sand, 1 g of P and 50 g of AMF for all concentrations (0, 50, 100 and 200 mM NaCl) and supplied with a nutrient solution in a completely randomized design. The results of this study showed that increased concentrations of NaCl in the culture medium significantly (P<0.001) decreased the leaf area, stem height, dry biomass, chlorophyll content, K, Ca and Mg from 100 mM NaCl. The total soluble proteins, proline, total free amino acids, soluble carbohydrates, total phenolic and flavonoids contents increased significantly (P< 0.001) from 50 mM NaCl in all treatments. The findings indicate that P and AMF positively influenced all the study parameters compared to the treatment with NaCl only. The use of (P + AMF) alleviated the toxicity of NaCl, improved growth, physiological and biochemical parameters and its usage could be encouraged for better development of crops in salinity affected areas.

Key words: *Solanum melongena*, phosphorous, arbuscular mycorrhizal fungi, growth parameters, metabolites, salinity.

INTRODUCTION

Salt affected areas are daily on the rise and the main causes are natural and anthropogenic factors. In Arid and semi-arid zones, salts from basal rocks move to the upper layer of the soil through the process of water

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evaporation or floods (Kosová et al., 2013; Hand et al., 2021), as a natural cause and over irrigation as the anthropogenic cause. In the coastal regions, it is caused by invasion of land by sea water due to wind and rain (Munns and Tester, 2008). Infact, the main salt responsible of salinization is NaCl and in general, 20% of irrigated land and 2.1% of dry land agriculture are facing salt problem (Munns and Tester, 2008; Hand et al., 2021). The accumulation of salts at the rhizosphere have a negative impact on plant growth and metabolism. Nutrient availability and uptake is affected and soon alters plant function, productivity, photosynthesis, ion homeostasis and antioxidants phenomena. These changes definitively limit crop production and hence the eventual death of the plant (Kosová et al., 2013; Nouck et al., 2016; Hand et al., 2021). Increasing the tolerance of crop plants to salinity will assist plants resist the negative effects of salinity and also improve their productivity in saline soils.

Phosphorous plays a significant role in plant growth. It is involved in important metabolic processes, such as photosynthesis, respiration, nucleic acid synthesis, membrane synthesis and nitrogen synthesis (Gulmezoglu and Daghan, 2017). The presence of high amounts of soluble salts in the soil affects the availability of phosphorous and other minerals to plants (Khan et al., 2018). It could either be due to the formation of insoluble complexes with metals like Fe and Al, or its existence in the organic form (Shahriaripour et al., 2011). The application of phosphorous (P) to plants in saline soils improves crop growth and consequent production. Nevertheless, beyond a certain level of salinity, enzyme activity is reduced (Shahriaripour et al., 2011).

Arbuscular mycorrhizal fungi (AMF) are soil microorganisms that contribute to the improvement of growth in several plant species under saline conditions (Evelin et al., 2009; Chun et al., 2018). It forms extensive networks which improve soil structure and also produce biochemicals like glomalin that enhance the absorption of water and nutrients, from the soil to the host plant tissues (Yang et al., 2008; Mahdi et al., 2010; Scagel and Bryla, 2017). AMF absorbs phosphorous (P) and other minerals (Al-Fahdawi and Allawi, 2019) from the soil and make them available to the host plant. When plants are subjected to saline stress, AMF ensures that the plants take up sufficient water and nutrients by adjusting osmotic pressure and mineral imbalance in the soil respectively. AMF plays an important role in membrane stability and stimulates plants to produce their own defense, enhance photosynthetic pigments and maintain the osmotic and ionic balance of the cell (Yang et al., 2008) and it has a high ability to secrete the phosphatase enzyme, which converts organic phosphorus into mineral phosphorus (Al-Fahdawi and Allawi, 2019). Scagel and Bryla (2017) and Nouck et al. (2022) showed that AMF in the presence of salinity decreased the Na^+ content and increased K, Ca, Mg, Na/K ratio and photosynthesis. Also, it has been reported that they modulate the biosynthesis of certain osmoprotectants such as proline, soluble carbohydrates, proteins and total free amino acids content improving and protecting the photosynthetic activity of plants (Yang et al., 2008; Scagel and Bryla, 2017).

The eggplant (*Solanum melongena* L.) is an economically important cash crop in the world with nutritional and medicinal properties and has great potentials as a food crop. Its global productions exceed 51.28 million tons/year (Kumar et al., 2020). It is a warm season crop, grown in various temperate and tropical parts of the world (Caruso et al., 2017) and cultivated primarily for its fruit. It provides significant nutritive and medicinal benefits due to the presence of vitamins, minerals, phenolics and antioxidants (Somawathi et al., 2015). It was hypothesized that arbuscular mycorrhiza and phosphorous influence the growth, nutrient uptake, chlorophyll content, and some metabolites of crop plants under saline stress. Therefore, the objective of this study was to evaluate the influence of arbuscular mycorrhizal fungi and phosphorous on the growth, nutrient uptake, chlorophyll content, biochemical constituents and non-enzymatic antioxidants of eggplant (*Solanum melongena* L. VAR. Yalo) under saline conditions.

MATERIALS AND METHODS

Study area and plant material

The research was carried out in a greenhouse located at New-Road Up-Station, Bamenda. The greenhouse was located at latitude 5° 56 North and longitude 10° 15 East, in Mezam division of the North West region of Cameroon. This area is located at 1614 m above sea level. The work was carried out from December 2019 to May 2020, average rain fall and temperatures are 865 mm/year and 30°C and relative humidity is close to 86%. Prevailing winds carry the tropical monsoon. The seeds of *Solanum melongena* Var. *yalo* used for the work and arbuscular mycorrhiza fungi (Bio 1: *Gigaspora margarita* + *Acaulospora tuberculata* and Bio 2: *Scutellospora gregaria*) were obtained from the Institute of Agronomic Research and Development (IRAD) Nkolbisson, Yaounde-Cameroon.

Plant growth conditions and salt treatments

Eggplant seeds were sterilized after a viability test with 3% of sodium hypochlorite for 10 min, washed ten times with demineralized water and transplanted into 3 L polythene bags previously filled with 3 kg of sterilized sand, with one plant each and five replications per treatment. The plants were arranged in a complete randomized block design and daily enriched with a modified nutrient solution (in g/L): Of 150 g $Ca(NO_3)_2$, 70 g KNO_3, 15 g Fe-EDTA, 0.14 g KH_2PO_4, 1.60 g K_2SO_4, 11 g $MgSO_4$, 2.5 g $CaSO_4$, 1.18 g $MnSO_4$, 0.16 g $ZnSO_4$, 3.10 g H_3BO_4, 0.17 g $CuSO_4$ and 0.08 g MOO_3 (Hoagland and Arnon, 1950). The pH of the nutrient solution was adjusted to 7.0 by adding HNO_3 0.1 mM. Plants were subjected to different salt concentrations (0, 50, 100 and 200 mM NaCl) with 0 mM NaCl as a control in the culture medium for a period of six weeks to determine the physiological and biochemical responses of cultivars to salt stress. The average day and night temperatures in the greenhouse were between 25

and 18°C, respectively during the growth period with average relative air humidity of 72%. Parameters were evaluated under greenhouse conditions: Stem height, leaf area, dry biomass of roots and shoots, chlorophyll (a+b), proline, soluble carbohydrates, total phenol and flavonoids content and mineral (Na, K, Ca and Mg contents) of roots and shoots.

Growth parameters

The leaf area, stem height, dry weights were recorded after six weeks. The leaf area was calculated using the formula, surface area (cm^2) = 1/3 (length × width) (Nouck et al., 2016). The eggplant parts were dried separately (roots and shoots) at 65°C for 72 h in an oven and their dry biomasses were determined (Nouck et al., 2016). The stem height was determined with a ruler.

Mineral distribution

The Pauwels et al. (1992) method was used to determined potassium, calcium, sodium and magnesium in roots and shoots. 2 g of dried organs were separately reduced to ashes by heating at 550°C for 4 h and thoroughly mixed with 250 mL of deionized water. The filtrate was analysed with an atomic absorption spectrophotometer (Rayleigh WFX-100).

Chlorophyll content

The Arnon (1949) method was used to determine chlorophyll (a+b) content. 0.80 g sample of fresh leaves were crushed and their contents extracted with 80% of alkaline acetone (v/v). The filtrate was analyzed using a spectrophotometer (Pharmaspec model UV-1700) at 645 and 663 nm wavelengths.

Metabolites

For the analysis of metabolites, all samples were obtained from the leaves.

Total soluble protein content

The total soluble protein content was determined using the Bradford (1976) method. An appropriate volume (from 0 - 100 µl) of crude extract was put into a test tube and the total volume was augmented to 100 µl with distilled water. 1 ml of Bradford solution was added to the sample. Then the mixture was thoroughly mixed with a vortex mixer. The absorbance was read at 595 nm with a spectrophotometer UV (PG instruments T60) after 2 min. The standard curve obtained was used to determine PR content.

Proline content

The proline content was estimated using Bates et al. (1973) method. 0.5 g of fresh leaves were weighed, crushed and put inside a flask. 10 mL of 3% aqueous sulphosalicylic acid was poured in the same flask. The mixture was homogenized, and then filtered with a Whatman N^o1 filter paper. 2 mL of filtered solution was poured into a test tube, and then 2 mL of glacial acetic acid and ninhydrin acid were respectively added into the same tube. The test tube was heated in a warm water bath for 1 h. The reaction was stopped by placing the test tube in an ice bath. 4 mL of toluene was added to the test tube and stirred. A purple-coloured mixture was obtained and its absorbance was read at 520 nm by spectrophotometer UV (Pharmaspec model UV-1700). The proline concentration was determined using the standard curve (µg/g FW).

Soluble carbohydrate content

The soluble carbohydrate content was obtained using phenol-sulphuric acid (Dubois et al., 1956). The fresh leaves (1 g) were ground in 5 mL of 80% ethanol and filtered with the Whatman N^o 1 filter paper. The extract was diluted with deionized water to make up 50 mL. 1 mL of sample was poured in test tube, followed by the addition of 1 mL of phenol solution and 5 mL of sulphuric acid. The mixture was then swirled. The absorbance was read at 490 nm using a spectrophotometer (Pharmaspec UV-1700 model). The quantity of CH was deduced from the glucose standard curve.

Total free amino acids content

The total free amino acids content was determined by the ninhydrin method (Yemm and Cocking, 1955). Fresh leaves (1 g) were ground in 5 mL of ethanol 80%, amino acids were then extracted using reflux technique in boiling ethanol for 30 min. After decanting, the supernatant was filtered using Whatman N°1 filter paper. The filtrate was collected and the residue used to repeat the extraction. The two filtrates were mixed and the raw extract of amino acid content was measured using ninhydrine method. The absorbance of purplish-blue complex was read at 570 nm wavelength. The standard curve was established using 0.1 mg/mL of glycine.

Flavonoids content

The flavonoids content of crude extract was determined by using the aluminum chloride colorimetric method (Chang et al., 2002). 1 mg/mL of the extract was prepared by dissolving 1 mg of eggplant leaves in 1 mL of ethanol. 50 µL of crude extract (1 mg/mL ethanol) was pipetted and 950 µL of methanol added to made it up to 1 mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution; 0.3 mL of 10% $AlCl_3$ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution was added, and the final volume of the mixture was brought to 10 mL with double-distilled water. The mixture was allowed to stand for 15 min, and absorbance was recorded on spectrophotometer (Pharmaspec UV-1700 model) at 510 nm. FLA content was calculated from a grutin calibration curve, and the result was expressed as grutin equivalent per gram dry weight.

Total phenolic content

The total phenolic content of the extract was determined by the Folin Ciocalteu method (Marigo, 1973). 1 g of fresh leaves were ground at 4˚C in 3 mL of 0.1 N HCl. After incubation at 4˚C for 20 min, the homogenate was centrifuged at 6000 g for 40 min. The supernatant was collected, the pellet re-suspended in 3 mL of 0.1 N HCl and centrifuged as previously. The two supernatants were mixed and 15 µL of the mixture was mixed with 100 µL Folin-Ciocalteu reagents and 0.5 mL of 20% Na_2CO_3 added before incubating at 40˚C for 20 min. After this time, the absorbance was read at 720 nm using a spectrophotometer (Pharmaspec UV-1700 model). A standard curve was established using chlorogenic acid. TP content was expressed as mg/g fresh weight.

Statistical analysis

The experiment was performed using a completely randomized

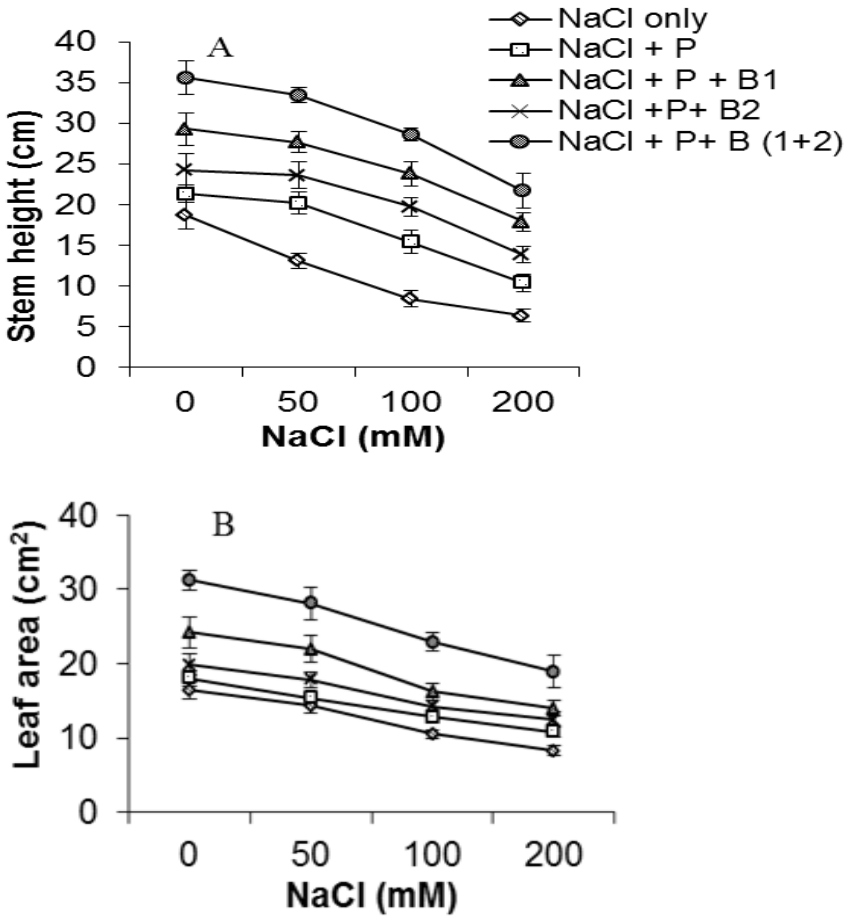

Figure 1. Effects of phosphorous and arbuscular mycorrhizal fungi on the growth parameters of *S. melongena* at different salt concentrations. A: Stem height; B: Leaf area. Bars are means (n= 5) ± SD.
Source: Author

design. All data were presented in terms of mean (± standard deviation), statistically analysed using Graph pad Prism version 5.01. The normality of data distribution was checked using the Shapiro-Wilk test (Shapiro and Wilk, 1965): The statistical difference between the groups and between the treatments was performed using the analysis of variance (ANOVA) and where the F- values were found to be significant, the treatment means were separated by Least Significant Difference (LSD) at 5% probability level using Duncan's Multiple Range Test (DMRT).

RESULTS

Plant growth

The growth parameters were generally affected with intake doses of NaCl in the culture medium (Figure 1). The treatment (NaCl + P) significantly ($P<0.05$) improved in all concentrations compared to the treatment with NaCl only. The treatment (NaCl + P+ B2) significantly ($P<0.01$) improved in all concentrations compared to the treatment with NaCl only and (NaCl + P). The treatment (NaCl + P+ B1) significantly ($P<0.01$) improved in all concentrations and above all treatments except the treatment (NaCl + P+ B (1+2)) which significantly ($P<0.001$) improved in all concentrations and treatments.

Dry weight partitioning

The dry weight of plant organs decreased significantly ($P<0.05$) in the culture medium with intake doses of NaCl for all treatments (Table 1) from 100 mM NaCl. The treatment (NaCl + P+ B (1+2)) significantly ($P<0.001$) improved in all concentrations compared to the treatment with NaCl only [(NaCl + P), (NaCl + P+ B2) and (NaCl + P + B1)]. For all treatments with NaCl + P+ AMF, a general improvement was observed in the dry biomass at all concentrations compared to treatments lacking AMF (NaCl and (NaCl + P). The interactions between AMF × NaCl and between AMF × NaCl × P were significant ($P<0.05$) in all treatments and concentrations except in shoots dry weight of treatment [(NaCl + P+ B (1+2)].

Table 1. Effects of phosphorous and arbuscular mycorrhizal fungi on the dry biomass of *S. melongena* at different salt concentrations.

Cultivar	Treatment (mM NaCl)	Dry weight (g)									
		NaCl only		NaCl + P		NaCl +B1		NaCl + B2		NaCl + B (1+2)	
		RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW
Yalo	0	1.3±0.05^{a}	2.77±0.07^{a}	2.03±0.08^{a}	3.74±0.09^{a}	3.07±0.02^{a}	6.63±0.82^{a}	2.45±0.04^{a}	5.01±0.48^{a}	3.61±0.19^{a}	7.05±0.35^{a}
	50	1.1±0.08^{a}	2.35±0.08^{a}	1.64±0.08^{a}	3.26±0.04^{a}	2.6±0.07^{a}	4.32±0.1^{b}	2.09±0.03^{a}	3.79±0.04^{b}	3.18±0.04^{a}	6.28±0.23^{b}
	100	0.81±0.06^{c}	1.35±0.05^{c}	1.15±0.06^{c}	2.13±0.08^{c}	1.89±0.19^{c}	2.48±0.16^{c}	1.42±0.08^{c}	2.13±0.02^{c}	2.62±0.08^{c}	4.64±0.24^{c}
	200	0.29±0.04^{d}	0.95±0.07^{d}	0.69±0.09^{d}	1.05±0.03^{d}	1.16±0.06^{d}	1.96±0.06^{d}	0.89±0.06^{d}	1.41±0.1^{d}	1.58±0.03^{d}	2.88±0.08^{d}
		ANOVA two way									
NaCl		*	*	*	*	*	*	*	*	*	*
P		*	*	*	*	*	*	*	*	*	
Biofertilizer		*	*	*	*	**	**	*	*	**	**
Biofertilizer × NaCl		*	*	*	*	*	*	*	*	*	**
Biofertilizer × NaCl × P		*	*	*	*	*	*	*	*	*	*

Mean results of five replications ± SD; within each column, mean followed by the same letter are not significantly different ($p < 0.05$). The asterisk indicates the interactions between treatment (* = $p < 0.05$ and **=$p < 0.01$).
Source: Author

Chlorophyll (a+b) content

The chlorophyll (a+b) content in the leaves of eggplant decreased significantly (P <0.001) from 50 mM NaCl for treatments with NaCl only and NaCl + P (Figure 2). The chlorophyll (a+b) decreased significantly (P <0.001) from 100 mM NaCl for treatments with NaCl + P+ B2, NaCl + P + B1 and NaCl + P+ B (1+2). The treatment (NaCl + P+ B (1+2)) significantly (P<0.001) improved in all concentrations compared to the other treatments (Figure 2).

Mineral distribution

The results of mineral uptake showed that Na^{+} increased significantly (P<0.001) with intakes doses of NaCl in the culture medium in plant partitions while others minerals (K^{+}, Ca^{2+} and Mg^{2+}) decreased significantly (P<0.001) at the same concentrations in all treatments (Figure 3). The treatment (NaCl + P+ B (1+2)) significantly (P<0.001), (P<0.01) and (P<0.05) improved in all concentrations compared to the treatment with NaCl only and NaCl + P, NaCl + P+ B2 and NaCl + P + B1 respectively in the culture medium (Figure 2).

Metabolites

Primary metabolites

The proline content (PRO), soluble carbohydrates (CH), total amino acids (FAA) and total soluble proteins (PR) of eggplant leaves increased significantly (P<0.001) with increased concentrations of NaCl in the culture medium for all treatments (Figure 4). The biochemical constituents of *S. melongena* increased significantly with increased NaCl concentrations. The PRO content results showed that the treatment (NaCl + P+ B (1+2)) significantly (P<0.001, P<0.01 and P<0.05) improved in all concentrations compared to the treatments with NaCl only and NaCl + P, NaCl + P+ B2 and NaCl + P + B1, respectively (Figure 4). The same pattern was observed with CH, FAA and PR at the same concentrations in the culture medium. In this study all treatments with AMF showed higher values than those without AMF (Figure 4).

Secondary metabolites

Total phenol content experienced an upward trend significantly (P<0.001) with increased salinity in all treatment. The treatment (NaCl + P+ B (1+2)) significantly (P<0.001, P<0.01 and P<0.05)

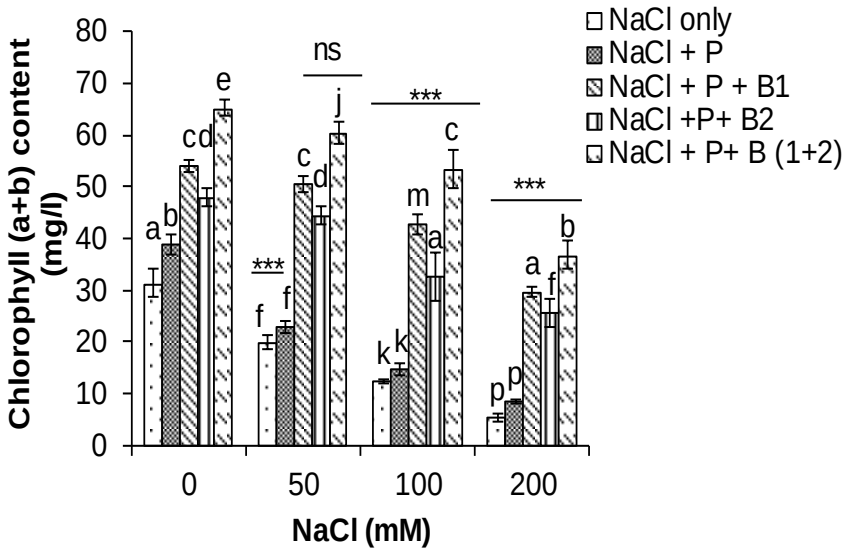

Figure 2. Effects of phosphorous and arbuscular mycorrhizal fungi on the chlorophyll (a+b) of *S. melongena* at different salt concentrations. Bars are means (n= 5) ± SD. Mean followed by the same letter are not significantly different ($p < 0.05$). The asterisk indicates the difference between the control vs treatment (ns= $p > 0.05$ and ***=$p < 0.001$).
Source: Author

improved in all concentrations compared to the other treatments in general. The results obtained for flavonoids content showed a similar pattern like those of total phenol content in the same treatments and concentrations (Figure 5).

DISCUSSION

The growth parameters generally decreased with intake doses of NaCl in the culture medium. These results corroborate those of Heidari et al. (2014). According to the study, the loss of water by plant cells caused the loss of cell turgor and shrinkage, reducing the rate of cell elongation. This contributed to the formation of shorter plant stems. In the same line, Kamran et al. (2020) in conformity with this trend reported that photosynthetic rates were retarded under high salinity due to decreased efficiency of growth hormones, resulting in decreased stem height. Heidari et al. (2014) and Kamran et al. (2020) justify the decrease in leaf area by stating that the loss of water by plant cells caused the loss of cell turgor and shrinkage, reducing the rate of cell elongation. Similar results were reported by Shahriaripour et al. (2011) on *Pistachio* seedlings. Even though their findings indicated that plant response to phosphorous addition under saline conditions greatly depended on the species and cultivar examined. Tang et al. (2019) also reported that stem height increased at low saline concentrations because of increased phosphorous mobility. Munns and James (2003); Shahriaripour et al. (2011) and Tang et al. (2019) revealed that P alleviates the negative impact of salinity on plant growth, particularly the leaf area of certain plants compared to those treated with NaCl only. For plants exposed to AMF in general, Evelin et al. (2009) showed that AMF alleviates the negative effects of salt stress by enhancing nutrient uptake, photosynthetic activities and water absorption; consequently contributing to the improvement of the growth of several plant species under saline conditions.

The dry weight of plant organs decreased in the culture medium with intake doses of NaCl for all treatments. Previous authors like Nouck et al. (2016) on *Lycopersicum esculentum* L. and Hand et al. (2017) on *Capsicum annuum* L. reported that plant dry biomass was subject to photosynthetic activity and mineral distributions. Inhibiting sufficient uptake of minerals to the tissues and a decrease in the photosynthetic efficiency of the plant led to a corresponding decrease in dry biomass. Additionally, Heidari (2012) explained that the reduced ability of plants to take in water under saline conditions caused slower growth. Also, excessive salts from the transpiration stream injured the cells of transpiring leaves. This is in line with our results for the treatment with NaCl only. The addition of phosphorus reflected an improvement in the results for all treatments and concentrations as compared with those of NaCl only. According to Tang et al. (2019), low Na^+ concentrations stimulate Na^+-dependent high affinity phosphate uptake which are involved in the synthesis of sucrose and starch during photosynthesis. Hence, an improvement in plant dry weight through the accumulation of dry matter. The

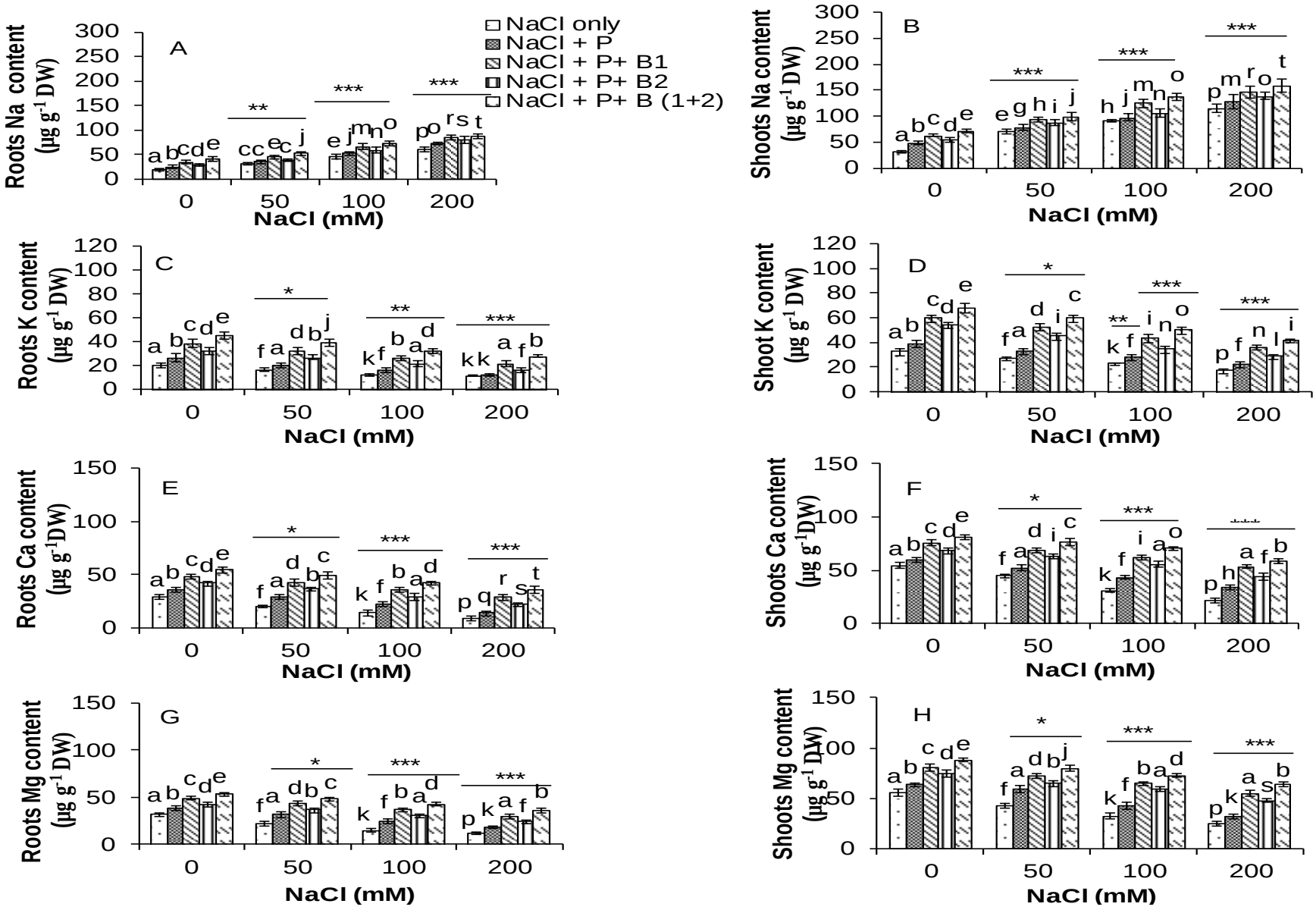

Figure 3. Effects of phosphorous and arbuscular mycorrhizal fungi on the mineral uptake of *S. melongena.* Bars are means (n= 5) ± SD. Mean followed by the same letter are not significantly different at ($p < 0.05$). The asterisk indicates the difference between the control vs treatment (*= $p < 0.05$, **=$p < 0.01$ and ***=$p < 0.001$).
Source: Author

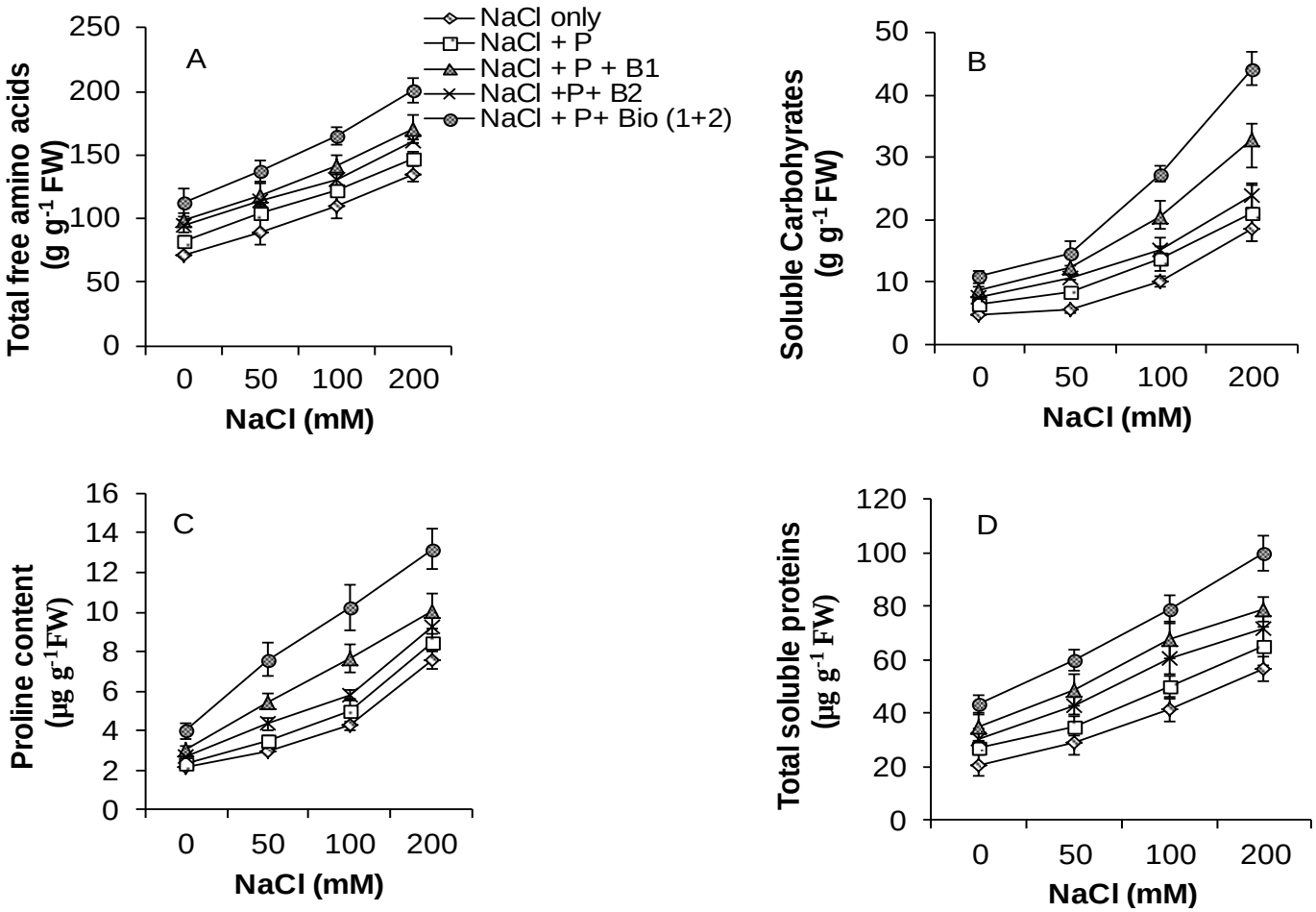

Figure 4. Effects of phosphorous and arbuscular mycorrhizal fungi on the primary metabolites of *S. melongena* at different salt concentrations. A: Stem height; B: Leaf area. Bars are means (n= 5) ± SD.
Source: Author

interactions between AMF + NaCl have a positive impact on plant biomass though the extent usually varies with the plant type and soil saline concentration. For all treatments with NaCl + P+ AMF a general improvement was observed in the dry biomass at all concentrations compared to treatments lacking AMF (NaCl and (NaCl + P). This could be due to the fact that AMF and P contribute to alleviate the detrimental effects of NaCl by enhancing the water uptake, mineral nutrition and photosynthetic activities of the plants. These changes were directly responsible for increased biomass (Chun et al., 2018). The interactions between AMF + NaCl + P tend to mitigate the adverse effects of NaCl on plant biomass, depending on the crop plant and saline concentration.

The chlorophyll (a+b) content in the leaves of eggplant decreased from 50 mM NaCl for the treatments NaCl only and NaCl + P. This result is in accordance with those of Murkute et al. (2006); Kaya et al. (2009) and Abdel Latef and Chaoxing (2011). They explained that the reduction in water potential, the antagonistic effects of NaCl on N absorption, the decreased uptake of certain nutrients like Mg needed for chlorophyll biosynthesis and the suppression of activity of specific enzymes required for the synthesis of photosynthetic pigments contribute to the decrease in chlorophyll. Yang et al. (2010) further reported that phosphorous application increased total chlorophyll content of plants by assisting in the production of photosynthetic pigments which are responsible for photosynthesis and also increase the stability of chlorophyll. This explains why treatment with NaCl + P improved compared to the treatment with NaCl only.

Several researchers such as Abdel Latef and Chaoxing (2011) and Nouck et al. (2022) indicated that AMF alleviate the negative effects of NaCl on chlorophyll content resulting in a higher chlorophyll concentration in plant leaf under saline conditions. The fungi are able to alleviate the antagonistic effects of Na^+ on Mg^{2+} uptake under salt stress (Giri et al., 2003). Our results agree with

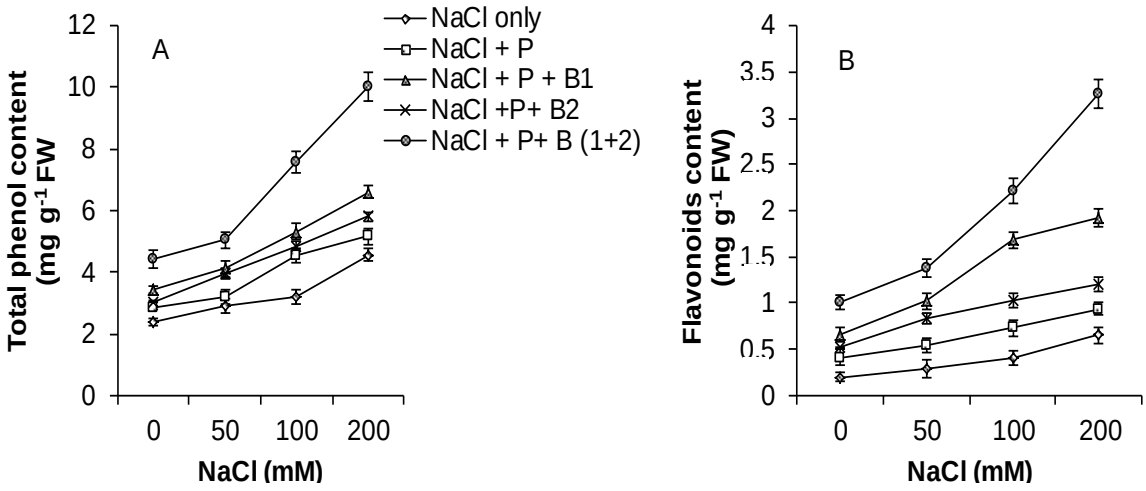

Figure 5. Effects of phosphorous and arbuscular mycorrhizal fungi on the secondary metabolites of *S. melongena* at different salt concentrations. A: Total phenol content; B: Flavonoids content. Bars are means (n= 5) ± SD.
Source: Author

the findings of Cekic et al. (2012). He reported that AMF and P were responsible for the high chlorophyll contents. AMF and P caused more uptake of Mg^{2+}; boosting the synthesis of chlorophyll, regulating the enzymes in chlorophyll synthesis while reducing chlorophyllase, maintaining cell membrane integrity and stability of the chlorophyll pigments and nutritional improvements. The significant improvement of chlorophyll in the treatment with NaCl + P + B (1+2) obtained can be justified by the type of biofertilizers used and the crop plant.

The Na^+ increased with intakes doses of NaCl in the culture medium in plant partitions while other minerals (K^+, Ca^{2+} and Mg^{2+}) decreased at the same concentrations in all treatments. According to Nouck et al. (2016) and Hand et al. (2017), the competition of Na^+ and K^+ for aerial plants resulted in greater accumulation of Na^+ in the shoots than in the roots. Ca^{2+} ameliorates salt stress through osmotic adjustments by enhancing ion uptake. In saline medium, Na^+ replace Ca^{2+}, resulting in the disintegration of cell membranes and cell walls. Although Na^+ increased with increased salinity in both shoots and roots while K^+ decreased, with the addition of phosphorous an improvement was recorded in the mineral uptake. The results of NaCl + P are in agreement with the works of Bargaz et al. (2016) and Gulmezoglu and Daghan (2017). Their findings suggested that the uptake of Na^+ is related to the concentration gradient, even in the presence of phosphorous. According to Bargaz et al. (2016), K^+, Ca^{2+} and Mg^{2+} work together with phosphorous to minimize the adverse effects of Na^+ on plants and the effect was expressed in the improved growth parameters of the plants.

The treatment (NaCl + P+ B (1+2)) improved in all concentrations compared to the treatment with NaCl only and NaCl + P, NaCl + P+ B2 and NaCl + P + B1 respectively in the culture medium. According to Giri et al. (2007) and Abdel Latef and Chaoxing (2011), the Na^+ concentration is lower in mycorrhizal than non mycorrhizal plants under salinity and could be explained by dilution effects of plant growth enhancement caused by AMF colonization. In this study, the negative effects of NaCl were attenuated by the association of phosphates and both AMF, enhancing the absorption of K^+, Ca^{2+} and Mg^{2+}. This led to the reinstatement of the ionic balance (Beltrano et al., 2013; Alqarawi et al., 2014) since K^+ and Ca^{2+} are involved in energy metabolism and Mg^{2+} is a central component of chlorophyll pigments. In addition the improvement in the uptake of these minerals also depends on the treatment, the type of plant, AMF and the mixture of AMF.

The primary metabolites increased with NaCl in the culture medium. It has been commonly reported by Kosovà et al. (2013), Nouck et al. (2016) and Hand et al. (2017) that biochemical constituents are salt-tolerance indicators and possess osmoprotective qualities. They showed that PR increased due to regulatory adjustments to stress, resulting in its active synthesis. This is because PR enhances plant salt tolerance. Its production is considered as an adaptive mechanism of plants to salinity. The findings of Nouck et al. (2016) indicated that the increase in FAA with increased salinity was due to the reduction of osmotic potential to maintain the turgid potential. According to Abdallah et al. (2016), osmotic stress caused by physiological drought is responsible for decreased osmotic potential, leading to the active synthesis and accumulation of CH. These results are consistent with those of Bargaz et al. (2016) and Gulmezoglu and Daghan (2017). They reported that soluble carbohydrates, total soluble proteins, total free amino acids and proline are produced in large amounts in response to salinity stress as an adaptive mechanism. This is because they function as osmoprotectants. The

treatment (NaCl + P) significantly (P<0.05) improved in all concentrations compared to the treatment with NaCl only. In this study, application of P was instrumental in the increase in photosynthetic pigments, which contributed to the significant increase in CH, PR, FAA and PRO in salinity stress.

This result indicates that the effect of AMF with P application constitutes one of the main reasons for increasing plant tolerance to salinity. According to Beltrano et al. (2013), salt-stress induces P deficiency in plants by reducing P uptake or translocation. This suggests that AMF enhances the plant growth by mechanisms that may not be related to improvement of P nutrition. Additionally, Al-Karaki (2000) and Giri et al. (2007) suggests that alleviation of salt stress by AMF includes improvement in P nutrition. They showed that improvement in P plant status is an important mechanism of salinity stress tolerance in mycorrhizal plants. In this work, all treatments containing AMF + P had elevated increase in primary metabolites as compared to the increase observed in plants treated with NaCl only. It is evident that the combination of P and AMF augment the production of primary metabolites in plants subjected to salinity stress. The quantity of primary metabolites produced is subject to crop type, type of biofertilizer and the mixture of biofertilizers.

Total phenol content and flavonoids experienced an upward trend with increased salinity in all treatments. Taïbi et al. (2016) maintain that the accumulation of total phenols and flavonoids are physiological responses to plant stress. They reduced oxidative damage by scavenging ROS, while maintaining chlorophyll levels and cell turgor to protect photosynthetic activities (Meguekam et al., 2014).

The quantity of TP and FLA in eggplants increased with the supply of P to the culture medium. In conformity with this result, Pontigo et al. (2018) explained that P improves plant efficiency to accumulate non-enzymatic antioxidants under stress conditions. It encourages the production of more non-enzymatic antioxidants so as to counter the effects of stress on the plants.

More flavonoids and total phenols were produced in eggplants that received P and AMF. In conformity with this result, Hashem et al. (2018) reported that AMF enhanced the accumulation of non-enzymatic antioxidants which assisted to counter the effects of stress on the plants. In our study, the mixture of P + Bio1 and Bio2 significantly improved non-enzymatic antioxidants compared to others treated with AMF+P. This could be due to the combined effect of 3 AMFs (Bio 1: *Gigaspora margarita* + *Acaulospora tuberculata* and Bio 2: *Scutellospora gregaria*).

Conclusion

From this study it can be concluded that Arbuscular mycorrhizal fungi associate with phosphorous to alleviate detrimental effects of salinity on the growth, nutrient uptake (higher Na^+ and lower K^+, Ca^{2+} and Mg^{2+} concentrations in leaf tissue), chlorophyll content and some metabolites of eggplant under saline conditions. The stem height, the leaf area, the dry biomass, the mineral uptake, the Chlorophyll (a+b) decreased with salinity doses of NaCl from 100 mM NaCl for the treatment with NaCl + P + AMF and from 50 mM for the treatment with NaCl only while osmolytes increased from 50 mM NaCl. The AMF + P enhanced the accumulation of all the study parameters than non-AMF + P plants under salinity. The addition of P, Bio 1(*Gigaspora margarita* + *Acaulospora tuberculata*) and Bio 2 (*Scutellospora gregaria*) in the culture medium caused significant increase in all the studied parameters and treatments. Phosphorous restrains the negative effects of salinity and associate with biofertilizers to mitigate the impact of salinity on the eggplants, resulting in significant improvements. The high accumulation of metabolites with salinity doses could be added as indicators of early identification and osmotic adjustment ability for salt-tolerant plants in salt stress conditions. Eggplants could be cultivated in soils with moderate salinity. Using AMF and P as an alternative way of decreasing NaCl stress in plants will be more beneficial as it maintains soil fertility.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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