Antimicrobial and antioxidant properties of African medicinal plants

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ABSTRACT

Objective: To study antioxidant and antimicrobial properties of Terminalia catappa, Psidium guajava, Alstonia boonei, Morinda lucida (M. lucida) and Spondias mombin leave extracts using ethanol, petroleum-ether and aqueous media.

Methods: The free radical 2, 2-diphenyl-1-picrylhydroazyl was used to measure scavenging activities of extracts, while phenolic and flavonoid content were estimated by spectrophotometry. Antibacterial screening of extracts was done by determining zone of inhibition using disc diffusion method. Plant extracts were tested against five strains of Gram-positive and Gram-negative bacteria. After sterilization, the discs were loaded with concentrations of broad spectrum ciprofloxacin, and prepared extract solutions of different concentrations were refrigerated for 24 h. Determination of minimum inhibitory concentration of extracts in the screening assay was according to micro-broth dilution, while determination of minimum bactericidal concentration was carried out by agar diffusion.

Results: The highest radical scavenging effect was found in petroleum ether extracts of all the plants with M. lucida and Psidium guajava having the highest and lowest values respectively. Aqueous solvent recorded highest phenolic content in all extracts with the exception of M. lucida. Flavonoid content was extracted better from Alstonia boonei and M. lucida with ethanol, while aqueous solvent extracted more from the other plants. With exception of extracts from petroleum ether, all others exhibited varying levels of antibacterial activities against E. coli, Pseudomonas aeruginosa, Salmonella typhi, Vibriocholera and Staphylococcus aureus.

Conclusions: This study proved that all crude extracts showed strong antioxidant and antimicrobial potentials, which qualified them as nutraceuticals in fish feed production.

1. Introduction

In the cause of treating fish diseases with antibiotics, about 80% of these drugs are released through the urinary and fecal excretion into the aquatic environment, this being the reason why the use of antibiotics in fish nutrition has been prohibited by the European Union, because of the consequent carries over effect of antibiotics to man[1]. The global demand for safe food and quest for eco-friendly environment has inspired the search for considerable development of natural alternatives to antibiotics such as herbs.

Natural products like medicinal plant[2], are rich in secondary metabolites compounds such as saponins, phenols, tannins, alkaloids, polysaccharides and polypeptides. They are possessed anti-oxidation and antimicrobial properties, hence there possible inclusion in animal feed.

Phytobiotics can be defined as plant derived products added to feed in order to better the animal state of health and improve the production performance of animal. In addition, medicinal plants are insecticidal, growth promoters, appetite enhancers and also stimulate the secretion of digestive enzymes among others[3]. The above properties have significant therapeutics actions against animal and human pathogenic organisms, viruses, bacteria and fungi[4]. Herbs are effectual in the treatment of the infectious diseases without side effects that are usually associated with synthetic antibiotics[5].

This study thus investigated the anti-oxidation and antimicrobial actions of leaves extracts from the following plants Morinda lucida (Oruwo) (M. lucida), Alstonia boonei (Ahun) (A. boonei), Spondias mombin (Iyeye) (S. mombin), Terminalia catappa (Tropical Almond) (T. catappa) and Psidium guajava (Guava) (P. guajava) using different solvents media.
2. Materials and methods

2.1. Collection of herbs

Samples of *M. lucida* (Rubiaceae), *A. boonei* (Apocynaceae), and *S. mombin* (Anacardiaceae) were bought at Oyingbo herbal market, Lagos, Nigeria, while tropical almond (*T. catappa*) and guava (*P. guajava*) leaves were collected around the University of Lagos, Akoka, Nigeria. All plant materials were taken to the Herbarium, Department of Botany, University of Lagos for identification. Plant materials were rinsed with clean water and spread evenly out and air dried at room temperature. The dried leaves of each plant were powdered using an electric blender. The following solvents were used for extraction, water, ethanol (95%) and petroleum ether (80°C).

2.2. Extraction process with aqueous solvent

Twenty grams of each dried plant samples was put into 250 mL conical flask containing 200 mL of distilled water. This was boiled at 75°C for 30 min. It was put on an orbital shaker for 24 h, filtered with muslin cloth and centrifuged at 10000 r/min for 5 min. The collected supernatant was concentrated using water bath at 75°C. A greasy final material, crude aqueous extract obtained from each herb was weighed, kept in labeled screw-cap bottles and refrigerated at 4°C.

2.3. Extraction process with ethanol

Twenty grams of each dried plant samples was put in 250 mL conical flask containing 200 mL of ethanol (95%). The mixture was put on an orbital shaker for 24 h and filtered with muslin cloth. The extraction was repeated twice, the filtrates obtained were put together, centrifuged at 10000 r/min for 5 min and the supernatant collected was concentrated using water bath at 70°C. A greasy final material, crude ethanol extract obtained from each herb was weighed, kept in labeled screw-cap bottles and refrigerated at 4°C.

2.4. Extraction process with petroleum ether solvent

Twenty grams of each dried plant samples was put in 250 mL conical flask containing 200 mL of petroleum ether (80%). The mixture was put on an orbital shaker for 24 h and filtered with muslin cloth. The extraction was repeated twice, the filtrates obtained were put together and centrifuged at 10000 r/min for 5 min. Finally solvent was removed from the extracts by heating in an oven at 40°C. A greasy final material, crude petroleum ether extract obtained from each herb was weighed, kept in labeled screw-cap bottles and refrigerated at 4°C.

2.5. Anti-oxidation assay

2.5.1. Evaluation of scavenging action of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals

The free radical scavenging activity of the fractions was measured in vitro by DPPH assay according to the following method[7,8]. A solution of 24 mg DPPH and 100 mL methanol was prepared to attain an absorbance of 0.98 ± 0.02 at 517 nm (using spectrophotometer) and refrigerated at 20°C until required. Three milliliter aliquot of the above solution was mixed with 100 µL of the sample at different concentrations (10–500 µg/mL). This mixture was vigorously shaken and incubated at room temperature in the dark for 15 min. Thereafter, the absorbance was read at 517 nm and the control assay was prepared as above without the sample. The estimation of the percentage DPPH radical scavenged was shown in the following equation: Scavenging effect (%) = (Value of control absorbance – Value of sample absorbance)/Value of control absorbance) × 100

2.5.2. Determination of total phenol

The spectrophotometric method[9] was used to determine the phenol content by mixing 1 mL of the sample with 1 mL of Folin-Ciocalteu’s phenol reagent. Five minutes later, 10 mL of 7% Na2CO3 solution was added, followed by the addition of 13 mL de-ionized distilled H2O and thoroughly mixed. At 23°C the mixture was kept in the dark for 90 min, after which the absorbance was read at 750 nm. The value of phenol was estimated by extrapolating the calibration curve which was made by preparing gallic acid solution. The value of phenol compound was read in triplicate and expressed as milligram of gallic acid equivalent (GAE) per g of dried sample.

2.5.3. Determination of total flavonoid

The total flavonoid compound of the extract was carried out by adding in a 10 mL test tube, 0.3 mL of sample, 3.4 mL methanol (30%), 0.15 mL NaNO2 (0.5 mol/L), 0.15 mL AlCl3.6H2O (0.3 mol/L) and 5 min later, 1 mL NaOH (1 mol/L) was added to the mixture[10]. The solution was thoroughly mixed, the absorbance was estimated against the reagent blank at 506 nm and the standard curve for total flavonoid was made using rutin standard solution (0 to 100 mg/L). The value of flavonoid compound was read in triplicate and expressed as milligram of rutin equivalent per g of dried sample.

2.6. Antibacterial assay

2.6.1. Microorganisms and standard inoculum preparation

The five microorganisms to which the five medicinal plant extracts were tested and obtained from the Microbiology Division, Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria. They were *Staphylococcus aureus* ATCC 25923 (*S. aureus*) (Gram-positive) while others were Gram-negative microbes: *Vibrio cholerae* ATCC 14035 (*V. cholerae*), *Escherichia coli* ATCC 25922 (*E. coli*), *Pseudomonas aeruginosa* ATCC 27854 (*P. aeruginosa*) and *Salmonella typhi* ATCC 700931 (*S. typhi*). The microorganisms were maintained on nutrient agar slant at 4°C with sub-culturing done every two months. Prior to their use for *in vitro* antimicrobial assay, a loop of each microbial culture on slant was placed in Mueller-Hinton broth (5 mL) and grown at 37°C for 6 h. The turbidity of the resulting broth culture was adjusted to 0.5 McFarland standards to give a cell density of 1.5 × 10⁸ CFU/mL and labeled as the standard inoculum.
2.6.2. Procedure for disc diffusion test

The antibacterial screening of the extract was evaluated by establishing the zone of inhibition using disc diffusion method[11]. The five plant extracts were tested individually against five pathogenic bacteria strains of Gram-positive and Gram-negative organisms by disc diffusion method[12].

2.6.3. Preparation of plant extracts solutions

The dried plant samples were individually weighed and dissolved in sterile distilled H₂O to prepare appropriate dilution to get different concentrations, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/mL which were refrigerated before they were used.

2.6.4. Inoculation of test plates

A total of 20 mL Mueller-Hinton agar were prepared in sterile Petri dishes (100 mm × 15 mm) and 100 μL of each standard inoculums of microorganism was plotted on the surface of the Mueller-Hinton agar plate using a sterile cotton swab (Hi media, readily prepared sterile swabs) which had been dipped into the turbid culture suspension. Whitman No. 1 filter paper was used to prepare discs of 6 mm in diameter and placed in hot air for sterilization. After which, the discs were loaded with different concentration of broad spectrum antibiotics, ciprofloxacin and plant extract solutions (T. catappa, P. guajava, A. boonei, M. lucida and S. mombin) of different concentrations and refrigerated for 24 h. Previously prepared paper discs were dispensed onto the surface of the inoculated agar plate, each disc was pressed down suitably apart and firmly to ensure complete contact with the agar surface was incubated at 5 °C for 1 hr to permit good diffusion and then transferred to incubator at 37 °C for 24 h. Afterwards, the plates were inverted and placed in an incubator set at 37 °C for 24 h.

2.6.5. Antibacterial screening of plant extracts

A total of 30 mg/mL of each plant extract was prepared, 20 μL of this extract was placed on the surface of sterile paper disc (6 mm) that was subsequently incubated at 4 °C for 15 min. Five extract discs were mounted on a plate and three replicate plates were used per extract. The plates were turned upside down and incubated at 37 °C for 24 h. The diameter of inhibition zone around each disc was estimated at the end of incubation period and the average of the triplicate tests was taken. The degree of action of the extracts was expressed in millimeters according to inhibition zone diameter; no activity (< 7 mm), active (7–11 mm), very active (> 12 mm).

2.6.6. Estimation of minimum inhibitory concentration (MIC)

The MIC of each plant extract identified in the screening assay was determined by micro-broth dilution method[13]. Serial dilutions of each extract at different concentrations, 0.5–32.0 mg/mL in sterile Mueller-Hinton broth were dispensed into the wells of 96-well microtitre plate (50 μL per well). This was followed by the addition of equal volume of each test microorganism at 10⁶ CFU/mL into the wells. The plate was covered with aluminum foil and incubated at 37 °C for 24 h. The MIC was the least concentration of the extract that produced no turbidity after 24 h of incubation[14]. The results were compared with standard antibiotic ciprofloxacin (10 μg/disc) from Oxford (UK).

2.6.7. Estimation of minimum bactericidal concentration (MBC)

The MBC of each active or very active medicinal plant was investigated by the agar diffusion method. About 10 μL aliquot of test bacteria culture was taken, from the microtitre plates used for the MIC assays. Each aliquot was used in the surface inoculation of Mueller-Hinton agar plate, which was incubated at 37 °C for 24 h. The MBC was regarded as the lowest concentration of the extract that produced no colonies of the test organisms after 24 h of incubation[14].

2.7. Statistical analysis

The experimental data were analyzed by One-way ANOVA using the SPSS package. Differences between means were determined and compared by Turkey’s test at 5% level of significance.

3. Results

The free radical scavenging action of the selected plant extracts using stable free DPPH was shown in Figure 1. The capability of different plant extracts to scavenging DPPH radicals and their values were presented in percentage (%). Extracts from petroleum ether differed significantly (P < 0.05) with the highest DPPH values compared with the other solvents. M. lucida has a DPPH value of 93.65 ± 0.64, S. mombin 87.75 ± 3.32, A. boonei 86.00 ± 2.83, T. catappa 62.25 ± 0.65 and the lowest value of 60.40 ± 0.42 for P. guajava aqueous extract (Figure 1).

![Figure 1. Free radical scavenging activity of aqueous, ethanol and petroleum ether extracts from leaf samples of T. catappa, P. guajava, A. boonei, M. lucida and S. mombin.](image-url)

The highest value (156.15 ± 1.63) for total phenolic content was recorded with aqueous extract of T. catappa, while the lowest total phenolic content of 87.85 ± 0.49 was recorded in P. guajava. There was no significant difference in the phenol content value recorded between the aqueous and petroleum ether extracts of A. boonei. Unlike other plants extracts, the M. lucida had the highest phenol content from the ethanolic solvent extraction with a value of 139.50 ± 0.71 (Figure 2).
The results of antimicrobial action of the plant extracts showed that the aqueous extract of *A. boonei* and aqueous *P. guajava* were active against all the five bacteria studied, with the least activity on *S. typhi* and *S. aureus* respectively as depicted by the value of the zone of inhibition (Table 1). On the other hand, ethanolic extract of *M. lucida*, aqueous extracts of *T. catappa* and *S. mombin* were active against four of the bacteria, with the exception of *S. aureus*, *V. cholera* and *E. coli*. However, antimicrobial action was recorded by the petroleum ether extracts of all the plant and the ethanolic extract of *T. catappa* against any of the bacteria studied.

From the antimicrobial activities screening extracts capable of clearing more of the microorganisms were selected for the minimum inhibitory and the minimum bacteria concentrations tests. The results of MICs of *A. boonei*, *M. lucida*, *S. mombin*, *T. catappa* and *P. guajava* on bacteria tested were shown in Table 2. The MIC of *A. boonei* (aqueous medium) had the highest MIC value of 15.50 ± 0.71 and the lowest value of 1.90 ± 0.14 with *V. cholera* and *E. coli*. The MIC of *M. lucida* (ethanolic medium) recorded the highest value (11.50 ± 4.95) with *P. aeruginosa* and the lowest value (7.75 ± 0.35) with *E. coli* whilst no action was recorded with *S. aureus*. Also, the MIC of *S. mombin* (aqueous medium) obtained the highest value (16.00 ± 0.00) with *P. aeruginosa* and the least value (5.75 ± 2.47) with *S. typhi* while no action was recorded with *E. coli*. The MIC of *T. catappa* (aqueous medium) obtained the highest value (15.50 ± 0.71) with *P. aeruginosa* and the least value (2.87 ± 1.23) with *E. coli* while *P. guajava* (aqueous medium) obtained the highest value (11.50 ± 4.95) on *S. aureus* and the least value (1.35 ± 0.64) with *E. coli* and *V. cholera*.

![Figure 2](image_url)

**Figure 2.** Total phenolic content of aqueous, ethanol and petroleum ether extracts from leaf samples of *T. catappa*, *P. guajava*, *A. boonei*, *M. lucida* and *S. mombin*.

The least value was recorded with petroleum ether flavonoid extraction in all plant samples, while the highest flavonoid content was obtained in aqueous extracts of *P. guajava* and *T. catappa*. With *M. lucida* and *A. boonei* the ethanolic extracts yielded more flavonoid content while no significant difference was observed among the different solvents used in the extraction of *S. mombin*. The least value of 45.50 ± 2.12 was obtained with the petroleum extract of *A. boonei* and highest flavonoid content of 126.20 ± 3.11 in *P. guajava* (Figure 3).

![Figure 3](image_url)

**Figure 3.** Total flavonoid content of aqueous, ethanol and petroleum ether extracts from leaf samples of *T. catappa*, *P. guajava*, *A. boonei*, *M. lucida* and *S. mombin*.

Table 1

| Extracts | Inhibition zone diameter (mm) |
|----------|-------------------------------|
|          | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| *E. coli* ATCC 25922 | 14 | G | G | 13 | 13 | G | G | 8 | G | 14 | G | G | 16 | 15 | G |
| *S. aureus* ATCC 25923 | 15 | G | G | 13 | 14 | G | G | 8 | G | 13 | G | G | 15 | 14 | G |
| *V. cholera* ATCC 14035 | 11 | G | G | 15 | 12 | G | G | 7 | G | G | G | G | 14 | 9 | G |
| *P. aeruginosa* ATCC 27853 | 12 | G | G | 14 | 13 | G | G | 8 | G | G | G | G | 13 | 10 | G |
| *S. typhi* ATCC 700931 | 14 | G | G | 13 | 13 | G | G | 8 | G | G | G | G | 15 | G | G |

Extract 1: Aqueous *A. Boonei*; Extract 2: Ethanol *A. Boonei*; Extract 3: Petroleum ether *A. Boonei*; Extract 4: Aqueous *M. lucida*; Extract 5: Ethanol *M. lucida*; Extract 6: Petroleum ether *M. lucida*; Extract 7: Aqueous *S. mombin*; Extract 8: Ethanol *S. mombin*; Extract 9: Petroleum ether *S. mombin*; Extract 10: Aqueous *T. catappa*; Extract 11: Ethanol *T. catappa*; Extract 12: Petroleum ether *T. catappa*; Extract 13: Aqueous *P. guajava*; Extract 14: Ethanol *P. guajava*; Extract 15: Petroleum ether *P. guajava*. G: The growths of microbes were observed.

The MBC was the least concentration of the extract that didn’t produced colonies of the test microbes after incubation for 24 h. The
MBC of A. boonei (aqueous extract medium) obtained the highest (31.00 ± 1.41) concentration among the five organisms tested with *V. cholerae* and the least value (6.00 ± 2.83) with *E. coli* and *S. aureus*. The MBC of *M. lucida* (ethanolic extract medium) recorded the highest value (23.00 ± 9.89) with *P. aeruginosa* and the lowest value (15.50 ± 0.71) with *E. coli* whilst no action was recorded with *S. aureus*. Also, the MBC of *S. mombin* (aqueous extract medium) obtained the highest value (32.00 ± 0.00) with *P. aeruginosa* and the lowest value (23.00 ± 9.89) with *S. aureus* while no action was recorded with *E. coli*. The MBC of *T. catappa* (aqueous medium) obtained the highest value (31.00 ± 1.41) with *P. aeruginosa* and the lowest value (5.75 ± 2.47) with *S. aureus* while no action was recorded with *V. cholerae*. The MBC of *P. guajava* (aqueous medium) obtained the highest value (23.00 ± 9.89) with *S. aureus* and the least value (2.78 ± 1.38) with *E. coli* (Table 3).

### Table 3

| Extract bacteria | A. boonei (AEC) | *M. lucida* (EEC) | S. mombin (AEC) | *T. catappa* (aqueous) | *P. guajava* (aqueous) |
|------------------|-----------------|-------------------|-----------------|------------------------|------------------------|
| *E. coli*        | 6.00 ± 2.83     | 15.50 ± 0.71      | -               | 7.75 ± 0.35             | 2.78 ± 1.38            |
| *S. aureus*      | 6.00 ± 2.83     | -                 | 23.00 ± 9.89    | 5.75 ± 2.47             | 23.00 ± 9.89           |
| *V. cholerae*    | 31.00 ± 1.41    | 24.87 ± 4.89      | 31.00 ± 1.41    | -                      | 6.00 ± 2.83            |
| *P. aeruginosa*  | 23.00 ± 9.89    | 23.00 ± 9.89      | 32.00 ± 0.00    | 31.00 ± 1.41            | 3.00 ± 1.41            |
| *S. rophi*       | 15.50 ± 0.71    | 16.00 ± 0.00      | 11.50 ± 4.95    | 15.50 ± 0.71            | 7.75 ± 0.35            |
|                  | - detected; AEC: Aqueous extract | EEC: Ethanol extract |

- No detected; AEC: Aqueous extract; EEC: Ethanol extract.

### 4. Discussion

Among the roles played by antioxidants include their defence against the reactive oxygen species, which are the injurious by-products generated during normal cell aerobic respiration[15]. Antioxidant could either be natural or from synthetic chemical source and have been studied widely to find protective compounds against a number of diseases that were due to oxidative stress and free radical-induced cell damage.

Antioxidant activities in plant materials could be measured through the DPPH activity, phenol or flavonoids content and the solvent used for polyphenolic extraction had significant effect on antioxidant activity.

The DPPH assay is often used in the investigation of the action of antioxidant to scavenge free radicals which are major factor in biological damage due to oxidative stress[16].

The diminution ability of DPPH radical is estimated by the reduction in its absorbance at 517 nm, caused by antioxidants, because as the reaction between antioxidant molecules and radicals in progress, it resulted in the scavenging of the radical by hydrogen donation changing the colour from purple to yellow.

The results from the study showed that all the solvents (ethanol, petroleum ether and aqueous) had the ability to extract antioxidant molecules with good potentials to act as free radical scavengers.

The phenol compound from plant have been shown to have multiple biological functions, including antioxidant activity, that is their defense against plant pathogens. Therefore, they are applied in the control of human and animal pathogenic infections[17,18]. Previous studies have shown good positive linear correlation between antioxidant potential and total phenol content in spices, herbs and other dietary plants. The phenolic compounds in plant extracts are more often associated with other molecules like proteins, polysaccharides, terpenes, chlorophyll and inorganic compounds. Hence, it requires suitable solvent for extraction of tannins[19]. In this study, the results of total phenol ranged from 86.5 ± 3.53 to 139.50 ± 0.70 mg GAE/g extract based on the different plants and solvents used showed their potentials as antioxidants that could be used in animal feed to prevent oxidative rancidity and replace commonly used synthetic antioxidants such as butylated hydroxyl anisole[20].

Reports from previous studies have shown that flavonoids extinguish active oxygen species and inhibit in vitro oxidation of low-density lipoproteins in addition to being anti-carcinogenic in several animal models[21-23], hence, this study showed that aqueous and ethanol were found to be better solvents for extraction of flavonoid compounds in the aforementioned plants.

The results of this study also showed that aqueous and ethanol extracts of all plant extracts, with the exception of ethanolic extract of *T. catappa* have varied antibacterial activities against the tested organisms. The petroleum ether extracts of all the respective plants had no inhibitory zone against all the tested organisms as shown in Table 1. This suggests that the aqueous and ethanol extracts of these plants are broad spectrum in their activities. Furthermore, the results of this study reflected the potent antimicrobial phytochemicals present in solvent extracts of the plant materials used and this correlates with the observation of previous works that plants contain substances such as flavonoids, phenols, triterpenoids and free hydroxyl groups that are very active antimicrobial compounds[24].

In the examination of antimicrobial potential of medicinal plants, the solvents that are usually employed are aqueous, methanol and ethanol[25,26]. The antimicrobial potentials of herbs against microbes examined in this study had their potency quantitatively and qualitatively determined by the presence or absence of inhibition zones, zone diameter, MIC and MBC values. The five plant extracts, relatively, exhibited significant antibacterial potentials against all the tested bacteria and the highest zone of inhibition obtained with the aqueous solvent in all plant extracts with the exception of *M. lucida* where the ethanolic extract had the best performance against the pathogenic bacteria.

Previous studies showed that tannins (polyphenols) act as growth inhibitors toward many microbes such as fungi, bacteria and yeasts by preventing the movement of nutrients into their cells thereby retarding their growth. Furthermore, when tannins form complex with microbial proteins or polysaccharides, the compounds formed are often irreversible, this characteristic confers bactericide and bacteriostatic properties on polyphenols[27]. From these results the plant extracts studied were relatively more effective against *S. aureus* compared to *E. coli*, *V. cholerae* and *P. aeruginosa*. The reason for this observation may be due to the cell walls of Gram-negative bacteria which are less permeable to antimicrobial compounds[28]. Therefore, with the plant extracts studied, the average antibacterial activity against the Gram-positive bacteria was slightly better than...
that of Gram-negative bacteria which was corroborated by earlier study[29].

This study showed that the above plants from Nigeria, sub-Saharan Africa possessed antioxidant and antimicrobial properties, which make them indispensable medicinal plants. Extraction with different solvents affected the yield of antioxidant and antimicrobial activities of all the five medicinal plants. The most efficient solvents for polyphenolic extraction were aqueous (water) and ethanol (90%) while petroleum ether extracts showed the least polyphenolic content. Therefore, practical application of these plant extracts may increase the shelf life of feed materials and improve health status of animals better than the synthetic preservatives that are currently used in the animal feeds.

Conflict of interest statement

We declare that we have no conflict of interest.

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