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Effect of Different Physical Factors on efficacy of *Thevetia Peruviana* leaf extract and bio-formulations

Bhanu Raj Meena *, Deepali Chittora, Sanjeev Meena, Tripta Jain, Kanika Sharma

Microbial Research Laboratory, Department of Botany, University College of Science, Mohanlal Sukhadia University, Udaipur, India

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ABSTRACT

Plant extract possess various secondary metabolites which are antifungal in nature and can be used as a safer alternative to the synthetic fungicides. As we all know that the chemical fungicides are harmful not only for humans but also for animals, other vegetation and for complete ecosystem. To overcome this problem, we have to focused on another alternative which are biologically libel and nonhazardous also. In the present study, herbal formulation was prepared in various combination ratios with *Thevetia peruviana* leaf extracts, cow dung and neem oil cake. The major aim of this short study is to check the stability of the said plant extracts and prepared herbal formulation on various physical factors like heat, temperature, pH, sunlight and storage etc. The extracts and herbal formulations were exposed to varying conditions of the parameters selected for a precise time period, and then observing the effect as a function of change in the crude extract activity, herbal formulation activity and change minimum inhibitory concentration of plant extract against the *Alternaria solani*. Control set of MIC, and extract free medium were maintained for comparison in each set of experiment against *Alternaria solani*. Results suggested that efficacy of leaf extracts and different formulations was not affected by wet heat up to 100 °C while slight reduction in antifungal activity of the plant extract and herbal formulations were observed with dry heat at 100 °C. In addition, slight reduction in activity of extract and herbal formulations was observed with change in pH. However antifungal activity of plant extract as well as herbal formulations, remain unaffected at alkaline pH (pH 9) and neutral pH (pH7). Storage for 6 and 12 months had no negative effect on extract and herbal formulation efficacy and the antifungal activity was observed similar to freshly prepared extract activity. The present study concluded that the plant disease or plant pathogens can be controlled by plant extract and plant based bioformulations by increasing the shelf life with some little changes in the physical parameters such as light, temperature, pH and storage.

1. Introduction

Various physical factors affect the amount of secondary metabolites such as alkaloids, flavonoids, sterols and tannins etc. present in the plant as which direct related to plant's therapeutic potential [1]. These secondary metabolites are responsible for the alteration of biochemistry and cytology of microbes and inhibitory activity of saponins on fungal growth has been reported [2]. Crude or alcohol extract of several plants have been screened for their possible antimicrobial activities against pathogenic virus, bacteria, fungi and protozoa [3]· Excess heating during extract preparation often affect biologically active heterogenous metabolites present in the plant extract which might influence their respective activity [4]. Any plant extracts and plant based herbal formulation or will be commercially viable if its stability can be maintained at varying physical conditions.Natural plant extract-based formulations need to be checked for efficacy and stability under various storage conditions; as a result can be employed for long term without alterations and deterioration [5].

Herbal formulations consist of intricate mixtures of various components obtained during extraction process. These components vary in their shelf-life, efficacy, concentration and stability. It poses a difficulty during determination or storage conditions because this is not as simple to resolve the stability issues associated with the resultant bio-formulations relay on the action and maintenance of action by a active content as well [6]. Bioactive compounds can degrade throughout the numerous stages of pre-treatment, processing [7,8]. Therefore, it is extremely imperative to study numerous storage factors like light, temperature, oxygen and pH to understand the stability of the active

* Corresponding author.
E-mail address: bhanurajmeena@gmail.com (B. Raj Meena).

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compounds of plant extract [9,10].

Many research works have been done on the stability of extract in the existence of diverse physical factors. The ability of active antimicrobial constituents of some Armenian herbs like *Agrimonia eupatoria, Hypericum alpestre, Rumex obtusifolius* and *Sanguisor baofficinalis* [11]. Temperature endurance was studied by using antimicrobial efficacy of plant decoction towards *Staphylococcus aureus* MDC 5233 just prior and latter of heat treatment. Some workers evaluated the antimicrobial efficacy of *Murrayakoengii* plant extract (root, leaf, bark) at different pH and temperature against bacterial species *E. coli, K. pneumoniae* and *Rhizobium*. Effect of osmolarity and pH on the antimicrobial efficiency of essential oils against pathogenic and food spoilage bacteria was also studied [12]. Effect of Temperature and pH on antimicrobial activity of *Tamarindus indica* Linn. against a number of frequent gram negative and gram positive bacteria and fungi was studied [13].

Heat treatments on antimicrobial actions of a variety of extracts of *Allium sativum* against certain bacteria and fungi was also studied by several workers [14].Some workers studied effect of temperature and expiry of toxicity during storage of *Citrus sinensis* oil [15]. The prerequisite conditions for use of plant extracts in such formulations therefore are that their physical and chemical properties should not undergo any drastic changes due to change in temperature, pH or exposure to sunlight and it should have a long shelf life. Present article describes the effect of various physical factors like sunlight, pH, heat and long-term storage on antimicrobial efficacy of leaf extract and herbal formulation prepared by the combination of *Thevetia peruviana* extracts, cow dung and neem oilcake in various ratios.

2. Materials and methods

2.1. Preparation of plant extract

Fresh plant leaves were collected from Botanical garden of College of Science, Udaipur. All plant parts were first cleaned with 0.1% $HgCl_2$ and then washed twice with distilled water. All plant parts were dark dried and mechanically powdered. Dried and powdered plant leaves were passed through sieve (mesh size 60) to get a fine powder which was further subjected to extraction process i.e. cold and hot extraction, using respective solvents.

2.2. Cold extraction

Crude extract was prepared by using the modified cold extraction method [16]. Cold extraction was prepared in water, 50% hydro alcohol and absolute alcohol. 20 gm of dried and powdered plant material was soaked in 100 ml of solvent (alcohol, water and 50% hydro alcohol) for 48 h. The decoction was filtered through Whatman filter paper no.1, vacuum dried with the help of a rotary vacuum evaporator. The dried residue was used as extract and solvent was recycled.

2.3. Hot extraction

step by step separation of various organic constituents present in dried plant material was done differentially by Reflux method of solvent extraction [17,18]. Series of different polarity solvent used for successive separation was as follows:

Petroleum ether → Benzene → Chloroform → Acetone → Alcohol →Methanol →Water.

This process includes continuous extraction of dried plant material in Soxhlet apparatus with a different polarity of organic solvents. In every step before extracting with next solvent the plant material is dried in an oven at temperature up to 50 °C. 40 gm. dry plant material was kept in Soxhlet extraction unit and extracted with 280 ml petroleum ether till all petrol soluble metabolites get extracted into solvent. Residue was dried and used in subsequent steps for extraction with next solvent. Same process was repeated with each solvent and lastly residue was macerated with chloroform water to attain aqueous fraction.

2.4. Preparation of herbal bio-formulation

Bioformulations prepared by combining active fraction of plant extracts, elicitor and binders were used for *in vitro* antifungal efficacy of against *Alternaria solani*.Best active plant extracts *i.e.* 100% alcohol crude extract, partially purified alcohol extract and best active elicitor and binder *i.e.* neem oil cake and gum acacia respectively were mixed in different combination to prepare different types of bioformulations.

Total 30 bio-formulation were prepared in which formulation no.1 to 15 were made by using 100% alcohol crude extract and formulation ratio number 16 to 30 were made by using partially purified alcohol extract in combination with elicitor and binder.

The efficacy of plant extracts and bioformulation were assayed under varying range of physical parameters like heat, temperature, pH, sunlight etc. for a specific period of time. Changes in the minimum inhibitory concentration (MIC) of partially purified extract, efficacy of crude extract and different bioformulation against the test organism were observed. A tube containing minimum inhibitory concentration of extract, bioformulation and without extract/bioformulation were maintained as a control in each set of experiments against *Alternaria solani*. In the present study 100% alcohol crude extract and partially purified alcohol extract of *Thevetia peruviana* leaves and best ratios of bioformulation [7,13,18,23] which was prepared by combining plant extracts, neem oil cake and cow dung were used for the experiments.

All ingredients of bioformulation were used in following ratio:

A. Herbal formulation ratio no. 7: (100% alcohol crude extract (6 ml): 100% Neem oil cake (2 ml): 100% cow dung (2 ml).
B. Herbal formulation ratio no. 13: (100% alcohol crude extract (3 ml): 100% Neem oil cake (4 ml): 100% cow dung (3 ml)).
C. Herbal formulation ratio no. 18: (Partially purified alcohol extracts (3 ml): 100% Neem oil cake (3 ml): 100% cow dung (4 ml).
D. Herbal formulation ratio no. 23: (Partially purified alcohol extracts (4 ml): 100% Neem oil cake (3 ml): 100% cow dung (3 ml).

These extracts and herbal formulations were found to be most potent and used for further experiments.

2.5. Effect of sunlight

The effect of sunlight on viability of the extracts and herbal formulation method were studied by this method. In this method sterile vials containing 5 ml of 100% alcohol crude extract, partially purified alcohol extract and herbal formulation (ratio no.7, 13, 18, 23) respectively were placed in sunlight for 15 h and 30 h. Then effect on the efficacy of extract and herbal formulation was assayed by the tube dilution method and poison food technique. In the poison food technique, 18 ml of molten PDA medium was poured into test tubes and then autoclaved. The molten sterilized medium along with 2 ml of extract/bioformulation was placed into petriplates after the solidification of the media, 6 mm inoculum disc of 7 day old culture of the fungus was aseptically inoculated upside down in the centre of the petriplate and incubated at 25 ± 2 °C.

On the 7th day of incubation average diameter of the fungal colonies was measured and percent mycelia growth inhibition was calculated by the following formula given below.

$$\% \text{ Mycelial growth inhibition} = \text{gc-gt/gc} \times 100$$

Where.

gc = Growth of mycelia colony after incubation period in control set subtracting the diameter of inoculum's disc

gt = Growth of mycelia colony after incubation period in treatment set subtracting the diameter of inoculum's disc

2.6. Effect of heat

Effect of dry heat was studied by exposing aseptic glass vials containing 100% alcohol crude extract, partially purified alcohol extract and bio-formulation (ratio no.7,13,18,23) to 40 °C and 90 °C for 4 hrs in a hot air oven while in case of wet heat; extract and bioformulation were kept at 50 °C and 100 °C in a water bath for 4 hrs [15]. Effect on the activity of extract and bioformulation was then assayed by twofold serial dilution method and poison food technique. One tube containing untreated extract as well as bio-formulation (room temperature) was maintained as a control for comparison.

2.7. Effect of pH

Effect of different pH *i.e.* 4, 7 and 9 on the efficacy of extract and bio-formulation was studied by this method [16]. Natural pH of extract and herbal formulation is 7.0.1 NHCl and 0.1 NaOH were used to change the pH to 4 and 9 in that order. Then culture medium was added to the tubes containing extract and herbal formulation and the tubes were inoculated with *Alternaria solani.* Inoculated tubes were then incubated at 27 ± 1 °C for 72 h and observed for the change in herbal formulation activity and MIC of the extract.

2.8. Effect of storage

Effect of storage on the antifungal activity of the extract and bio-formulation was assayed by method suggested by this method [17]. Extracts and herbal formulations were store at room temperature (25–27 °C) in glass bottle with change in their activity was assayed at regular intervals of 6 month up to 24 months by tube dilution method.

3. Results and observations

Effect of various physical factors *viz*. heat, sunlight, pH and long-term storage on antifungal activity of plant extracts and herbal formulations were given in Table. 1 – 4 and Table. 5 – 8.

Results of effect of wet and dry heat on extracts and different formulations are given Table no. 1 and Table no.5 respectively. Results suggested that efficacy of leaf extracts and different formulations was not affected by wet heat up to 100 °C while slight reduction in antifungal activity of both the plant extract and herbal formulations were observed with dry heat at 100 °C. There was no change in activity due to storage time and sunlight treatment in both the extracts and herbal formulations (Table no. 2 and 3, Table no. 6 and 7).

Table no.4 and Table no. 8 depict the effect of pH on the efficacy of plant extract and herbal formulation. Slight reduction in activity of extract and herbal formulations was observed with change in pH. This reduction was observed at acidic pH (pH 4) at which slight growth of test fungus, *Alternaria solani,* was observed. Antifungal activity of both plant extract and herbal formulations, remain unaffected at alkaline pH (pH 9)

Table 1
Effect of heat on antifungal activity of crude and partially purified leaf extract of *Thevetia peruviana* against *Alternaria solani*.

S. No.	Extracts	Dry heat			Wet heat		
		R.T.	50 °C	100 °C	R.T.	50 °C	100 °C
1.	100% Alcoholic crude extract	13.00 ± 1.00 (mm)	13.00 ± 1.00	18.00 ± 1.00	13.00 ± 1.00	13.00 ± 1.00	13.00 ± 1.00
2.	Partially purified Alcohol extract	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml
3.	Control (without extract)	80.66 ± 0.57					

R.T. - Room Temperature.

Table 2
Effect of storage on antifungal activity of crude and partially purified leaf extract of *Thevetia peruviana* against *Alternaria solani*.

S. No.	Extracts	6 months	12 months	Fresh extract
1.	100% Alcoholic crude	13.00 ± 1.00	13.00 ± 1.00	13.00 ± 1.00
2.	Partially purified alcohol	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml
3.	Control (without extract)	80.66 ± 0.57		

Table 3
Effect of sunlight exposure on antifungal activity of crude and partially purified leaf extract of *Thevetia peruviana* against *Alternaria solani*.

S. No.	Extracts	15 h	30 h	Unexposed condition
1.	100% Alcoholic crude	10.00 ± 0.577	10.00 ± 0.577	10.00 ± 0.577
2.	Partially purified alcohol	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml
3.	Control (without extract)	80.66 ± 0.57		

Table 4
Effect of pH on antifungal activity of crude and partially purified leaf extract *Thevetia peruviana* of against *Alternaria solani*.

S. No.	Extracts	pH4	pH9	Control (pH 7)
1.	100% Alcoholic crude	13.00 ± 1.00	15.00 ± 1.00	13.00 ± 1.00
2.	Partially purified alcohol	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml
3.	Control (without extract)	80.66 ± 0.57		

Table 5
Effect of Heat (Dry and Wet) on antifungal activity of herbal formulations.

Herbal formulations ratio number	Dry heat			Wet heat		
	R.T. (Growth Diameter in mm)	50 °C	100 °C	R.T.	50 °C	100 °C
7	10	10.66	16.66	10.66	10.66	10.66
13	10	10.66	17.66	10.66	10.66	10.66
18	10.22	9.23	11.66	10.66	10.66	10.66
23	10.22	9.32	13.66	10.66	10.66	10.66
Control (Without extract)	80.66					

Table 6
Effect of Storage period on antifungal activity of herbal formulations.

Herbal formulation ratio number	6 months	12 months
7	10	10
13	10	10
18	10.007	10
23	10	10
Control	80.22	

Table 7
Effect of Sunlight on antifungal activity of herbal formulations.

Herbal formulation ratio number	15 h sunlight exposure	30 h sunlight exposure	Control condition
7	10.22 ± 0.577	11.23 ± 0.577	10.22 ± 0.577
13	10.22 ± 0.577	11.23 ± 0.577	10.22 ± 0.577
18	10.22 ± 0.577	11.23 ± 0.577	10.22 ± 0.577
23	10.22 ± 0.577	11.23 ± 0.577	10.22 ± 0.577
Control	80.66		

Table 8
Effect of pH on antifungal activity of herbal formulations.

Herbal formulation ratio number	pH4	pH9	Control/neutral pH (pH 7)
7	12.23	10.22	10.22
13	12.23	10.22	10.22
18	12.23	10.22	10.22
23	12.23	10.22	10.22
Control	80.66		

and neutral pH (pH7).

4. Discussion

Use of herbal formulations is safe and cheaper method for controlling the plant diseases without compromising environmental and human health. The benefits of these herbal formulations only will be long-lasting when it will be remaining unchanged in their activities so these formulations need to be assayed for stability under various physical conditions. Commercial viability of these formulations depends on stability in varying environmental conditions and good shelf life without any effect on antimicrobial efficacy. The stability is aimed at assuring that the active molecule/fraction leftovers within the specifications established to make sure its characteristics, potency, superiority and purity. It can be summarized as the duration of time in precise conditions of storage that a product will remain active within the pre-defined confines for all its significant features. Environmental factors such as temperature, sunlight, heat, pH and storage circumstances can affect the stability of these plant based formulations hence, need to be studied for prolonged stability [18].

Results suggested that sunlight do not affect the antimicrobial efficacy of plant extracts and herbal formulations. Reason may be the active compounds did not undergo photochemical degradation in the occurrence of sunlight. Hence no structural modifications of functional groups of compounds take place which are required for the antimicrobial activity. Thus results suggest that active molecules of *Thevetia peruviana* leaf extract are light stable. Some workers reported that visible light activates the bioactive constituents in the plant extracts and therefore enhances in the antifungal efficacy of the extracts against toxigenic *Aspergillus flavus* which causes aflatoxin production [19].

Among the wet and dry heat treatment, wet heat up to 100 °C did not affect the antifungal activity whereas dry heat up to 100 °C leads slight reduction in antifungal activity. The utilization of waterless heat is destructive and this leads to destruction of active compounds which are no more available to take action against fungal pathogens. Some researchers observed that dry heat leads to reduction in antimicrobial activity whereas moist heat did not affect antifungal activity of plant extract [20]. Ranglovát *et. al.*, (2015) found that heat treatment leads reduction in antimicrobial activities of Garlic (*Allium sativum*) [21].

In case of change in pH antifungal activity of plant extract and herbal formulations prepared using *Thevetia peruviana* leaf powder was observed. However, antifungal activity of extract and herbal formulations remained same at neutral pH as well as alkaline pH [22]. found that alkaline pH was observed to be best for both growth as well as antimicrobial activity. Maximum antifungal activity of *Streptomyces* was observed at neutral pH [23,24] Some workers described that methanol extract from peanut hulls had a superior antioxidant efficacy at neutral and acid pH [25]. The antioxidant efficacy of different extracts from cocoa by-products was elevated at alkaline pH. Yen*et al.*, 1993 found that anise oil exhibited superior antifungal efficacy at pH 4.8 than at pH 6.8, while oil of *Cedrus deodara* is most active at pH-9. Yen e*t al.,* 1993 described that a methanol extract from peanut hulls had a superior antioxidant efficacy at neutral and acid pH [26]. Shittu*et al.*, 2007 studied that anise oil exhibited superior antifungal efficacy at pH 4.8 than at pH 6.8, whereas oil of *Cedrus deodara* is most active at pH-9 [27].

Long term storage condition has no effect on antifungal efficacy of *Thevetia peruviana* plant extract and herbal formulations. Similar results was observed by some workers [12].

Results suggested that extremes of dry heat and acidic pH affect the antifungal efficacy of extracts and formulation prepared from *Thevetia peruviana* leaf. Other factors like storage condition, moist heat and sunlight didn't alter the inherent antifungal property of extracts and herbal formulations. Thus prepared formulation and extract employed in this study possess satisfactory physical stability which is most important criteria need to be satisfied for commercialization.

5. Conclusion

Current study investigates the overall storage stability of test plant extract and herbal formulations based on varying physical condition. Stability was measured in terms of existence of antifungal property under extremes of different environmental condition. Since the extract remains stable at alkaline/neutral pH and temperature extremes it can be store for 6 months without any loss in its antimicrobial properties which is very beneficial characteristics of formulations and extract for commercialization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] Surbhi& Sharma Mehta, Kanika, Effect of plant extract on cytomorphological alteration in *Alternaria solani*, Acta Scientific PharmaceuticalSciences (5) (2021) 38–45.
[2] Mediani, Ahmed, FaridahAbas, Chin Ping Tan, AlfiKhatib, Effects of different drying methods and storage time on free radical scavenging activity and total phenolic content of cosmos caudatus, Antioxidants 3 (2) (2014) 358–370.
[3] Malkhan Gurjar, ShahidAkhtar Ali, Masood, Singh, Kangabam, Efficacy of plant extracts in plant disease management, Agric. Sci. 03 (2012) 425–433.
[4] J.U. Mollah, W. Islam, Toxicity of *ThevetiaPeruviana* (pers) schum. Extract to adults of *CallosobruchusMaculatus* F. (*Coleoptera: bruchidae*), J. Agric. Rural Dev. (2007) 105–109.
[5] DeepaHada&Kanika Sharma, Effect of different physical factors on *Cassia fistula* fruit pulp extract and their herbal formulation efficacy, Juniper Publishers Inc. Global Journal of Pharmacy & Pharmaceutical Sciences 4 (2) (2017) 24–29.
[6] V.N. Gunnlaugsson, M. Thakur, E. Foras, H. Ringsberg, Ø. Gran-Larsen, S. Margeirsson, EPCIS Standard Used for Improved Traceability in the Redfish Value chain, International Conference on Modern Information Technology in the Innovation Processes of the Industrial Enterprises, 2011, pp. 182–192.
[7] I. Ioannou, I. Hafsa, S. Hamdi, C. Charbonnel, M. Ghoul, Review of the effects of food processing and formulation on flavonol and anthocyanin behaviour, J. Food Eng. 111 (2) (2012) 208–217.
[8] S.Y. Lee, A. Mediani, A.H. NurAshikin, A.B.S. Azliana, F. Abas, Antioxidant and α-glucosidase inhibitory activities of the leaf and stem of selected traditional medicinal plants, Int. Food Res. J. 21 (1) (2014).

[9] M.W. Kearsley, N. Rodriguez, The stability and use of natural colours in foods: anthocyanin, β-carotene and riboflavin, Int. J. Food Sci. Technol. 16 (4) (1981) 421–431.
[10] R.K. Sharma, A. Goel, A.K. Bhatia, *Lawsoniainermis* Linn: a plant with cosmetic and medical benefits, Int. J. Appl. Sci. Biotechnol. 4 (1) (2016) 15–20.
[11] M. Ginovyan, M. Petrosyan, A. Trchounian, Antimicrobial activity of some plant materials used in Armenian traditional medicine, BMC Compl. Alternative Med. 17 (1) (2017) 1–9.
[12] P. Yadav, M. Mirza, K. Nandi, S.K. Jain, R. Kaza, P. Sharma, A. Saxena, Clinical significance of circulating microRNA-200c expression in breast cancer, Eur. J. Cancer (103) (2018) 97.
[13] P.O. Angienda, D.J. Hill, The effect of sodium chloride and pH on the antimicrobial effectiveness of essential oils against pathogenic and food spoilage bacteria: implications in food safety, WASET 57 (2011) 1033–1038.
[14] N. Canillac, A. Mourey, Effects of several environmental factors on the anti-Listeria monocytogenes activity of an essential oil of Piceaexcelsa, Int. J. Food Microbiol. 92 (1) (2004) 95–103.
[15] Mamta Patra, Sushil K. Shahi, Anupam Dikshit, Utilization of pericarp of *Citrus sinensis* oil for the development of natural antifungal against nail infection, Curr. Sci. 84 (12) (2003) 1512–1515.
[16] Shadomy, Ingraff, in: E.H. Lennet, E.H. Spauling, J.peds Truant (Eds.), A Mannal of Clinical Microbiology, *American Society of Microbiology*Washigton, 1979, p. 569.
[17] J.B. Harborne, Methods of plant analysis, in: Phytochemical Methods, Chapman and hill, London, NewYork, 1984, pp. 5–6.
[18] C.K. Kokate, A Text Book of Practical Pharmacognosy, fifth ed., New Delhi, VallabhPrakashan, 2005, p. 107.
[19] J.H. Oughari, Antimicrobial activity of *tamarindusindica* Linn, Trop. J. Pharmaceut. Res. 5 (2) (2006) 597–603.
[20] J.M. Olson, S. Vongpunsawad, H. Kuivaniemi, A. Ronkainen, J. Hernesniemi, M. Ryynänen, G. Tromp, Search for intracranial aneurysm susceptibility gene (s) using Finnish families, BMC Med. Genet. 3 (1) (2002) 1–7.
[21] K. Ranglová, P. Krejčová, R. Kubec, The effect of storage and processing on antimicrobial activity of *Tulbaghiaviolacea*, South Afr. J. Bot. 97 (2015) 159–164.
[22] N. Rønsted, M.R. Symonds, T. Birkholm, S.B. Christensen, A.W. Meerow, M. Molander, A.K. Jäger, Can phylogeny predict chemical diversity and potential medicinal activity of plants? A case study of Amaryllidaceae, BMC Evol. Biol. 12 (1) (2012) 1–12.
[23] M.L. Motsei, K.V. Lindsey, J. Van Staden, A.K. Jäger, Screening of traditionally used South African plants for antifungal activity against Candida albicans, J. Ethnopharmacol. 86 (2–3) (2003) 235–241.
[24] Y.M. Zhang, L.Y. Zhang, K.Q. Wang, J.B. Ge, Distribution of angiotensin converting enzyme gene polymorphism among Northern Hans, Dahurs, and Ewenks, Acta Pharmacol. Sin. 22 (8) (2001) 747–750.
[25] R. Kloosterman, J. Rath, Immigrant entrepreneurs in advanced economies: mixed embeddedness further explored, J. Ethnic Migrat. Stud. 27 (2) (2001) 189–201.
[26] C.S. Kumari, S. Govindasamy, E. Sukumar, Lipid lowering activity of *Ecliptaprostrata* in experimental hyperlipidemia, J. Ethnopharmacol. 105 (3) (2006) 332–335.
[27] L.M. Njoki, S.A. Okoth, P.M. Wachira, Effects of medicinal plant extracts and photosensitization on aflatoxin producing *Aspergillusflavus* (Raper and Fennell), Int. J. Microbiol. 17 (2017) 1–9.