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Table of Content

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Full Length Research Paper

Evaluation of the effects of plant aqueous extracts as anti-diabetic agents on alloxan induced diabetic male rabbits

Hadia Akber Samoo*, Jagarwanti Maheshwari, Atta Muhammad Babar, Ather Hameed Sheikh, Imrana Khushk, Muhammad Aqeel Bhutto, Jawaid Ahmad Khokhar, Abdul Sattar Qureshi, Syed Akbar Shah and Aftab Hussain Khaskheli

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This study investigates the effect of barley *(Hordeum vulgare)*, black plum *(Syzygium cumini)* and Chinese tree leaves (*Pistacia chinesis*) aqueous extracts on serum glucose, cholesterol, urea, uric acid level and body weight of normal and alloxan-induced diabetic male rabbits. Rabbits were kept in fasting condition for 12 h, then diabetes was induced using alloxan monohydrate (150 mg/Kg). From the 7th day of diabetes induction, blood serum was collected for glucose analysis. Rabbits having fasting glucose level ≥ 250 mg/dL were considered as diabetic. Rabbits were treated with 1% barley, black plum and Chinese tree aqueous extract to check their effects on the serum glucose, serum cholesterol, serum urea, uric acid and body weight of the animals. Samples were collected after every 72 h for biochemical analysis. It was noted that 1% barley, black plum and Chinese tree aqueous extract reduced 43.92, 39.05 and 32.47% glucose level. Results suggest that oral administration of aqueous extract of barley, black plum and Chinese tree reduced glucose, cholesterol, urea, and uric acid level. Therefore, these medicinal plants should be supplemented as herbal drugs in the treatment of various complex diseases and specially to control diabetes.

Key words: Chinese tree leaves, antidiabetic agents, barley, black plum, rabbits, diabetes mellitus.

INTRODUCTION

Diabetes mellitus is one of the chronic diseases that occur either due to decrease in insulin secretion or lack of insulin peripheral activity (Mutalik et al., 2003); it approximately causes half of all deaths occurring at the age of 70. Literature reports that 415 million adults were living with diabetes in 2015 and this would increase to 642 million by 2040 (Rahelic, 2016). Diabetic complications include increased gluconeogenesis and ketogenesis (Kumar et al., 2011) and increased risk of

heart attacks and strokes (Jousilahti et al., 2010). Diabetes mellitus has two types, Type 1 occurs due to β-cell destruction, and is mostly insulin dependent diabetes (Alberti and Zimmet, 1998); type-2 is triggered by mutation in β-cell gene, and is the most common forms of adulthood onset diabetes caused by change in glucokinase and HNF-1 alpha genes. Type2 diabetes remains undiagnosed because hyperglycaemia does not show any symptoms; such patients may develop micro

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or macrovascular complications (Chisholm et al., 1997; Lillioja et al., 1993).

Medicinal plant has been used for the cure of diabetes mellitus before the invention of insulin therapy in 1921 (wadood et al., 2007). Medicinal plants are good sources of hypoglycemic compounds and these plants are used as adjuncts to the existing therapies for the treatment of diabetes mellitus (Sharma et al., 2008). Plants' extract has effect on blood glucose level through different mechanisms: some plants have insulin-like compounds, some might inhibit insulin activity (Hussain et al., 2013) and other mechanisms could enhance the regeneration of the β-cells in the pancreas (Hosseini et al., 2015). More than 400 medicinal plants have been used for the treatment of diabetes mellitus, and screened for anti-diabetic agents (Thomson, 2006). Various parts of plant such as fruit, seeds, and leaves have been reported for the treatment of type 2 diabetes (Mahesar et al., 2010, Khushk et al., 2010). Bitter melon (*Momocardia charantia*), karela is a member of the cucumber family grown for its edible fruit. Bitter melon decreases the level of glucose in the blood (Dans et al., 2007). Presently, there is growing interest in herbal remedies due to the side effects associated with the oral hypoglycemic agent (therapeutic agent) used for the treatment of diabetes mellitus.

Type 2 diabetes can be controlled by taking *M. charantia* capsules over a period of three months (Dans et al., 2007). Corn silk (*Zea mays*) is mostly used in China against diabetes (Mendoza-López et al., 2017). Various ether soluble fractions of onion (*Allium cepa* L.) and soluble fractions of dried onion powder show anti hyperglycemic activity against diabatic rabbits. Onion (*A. cepa* L.) is also known to have anti-oxidant and hypolipidemic activity (Jain and Vyas, 1974).

Garlic (*Allium sativum*) is a member of the Liliaceae family, which is one of the most popular herbs used worldwide to reduce various risk factors associated with several disease (Thomson, 2006). Garlic has been found to be effective in lowering serum glucose levels in STZ-induced as well as alloxan-induced diabetic rats and mice. Most studies showed that garlic can reduce blood glucose levels in diabetic mice, rats and rabbits. Some hypoglycemic activity was found in garlic as compared to diabetic control groups (Nadir Ali et al., 2013).

Soybean (*Glycine max*) belongs to the family Leguminosae or Fabaceace, which contains vegetable protein, oligosaccharides, dietary fiber, phytochemicals and minerals. Soybean reduces coronary heart disease and lowers cholesterol level. Also, it has anti-inflammatory and anti-carcinogenic effect on digestive system (Aparicio et al., 2008). In Pakistan, soybean is cultivated for oil extraction (Malik et al., 2006).

Barley (*Hordeum vulgare*) is a rich source of magnesium, a mineral composed of more than 300; enzymes, including those involved in glucose metabolism and insulin secretion. Black plum has been used for

centuries as anti-diabetic medicines. Barley has gained popularity in recent years owing to its association with the soluble β-glucan fiber and the phytochemical compounds it contains with various health benefits (Alu'datt et al., 2012). Epidemiological studies have associated the regular consumption of barley with its potential to reduce the risk of certain diseases, such as chronic heart disease (Sullivan et al., 2013), colonic cancer (Dongowski et al., 2002), high blood pressure (Behall et al., 2006), and gallstones (Hoang et al., 2011). Reports of barley focused on maintaining a healthy colon (Kanauchi et al., 1999), inducing immune stimulation (Tada et al., 2009), and generally boosting the immune system (Kemp et al., 2013). Barley extract has not been evaluated for its antidiabetic efficiency, thus in present study, barley extract was found to be the best antidiabetic agent against alloxan induced rabbits. Chinese tree (*Pistacia chinesis*) has been used for medicines (TCM) and local herbal crops for abdominal sickness, abscesses, amenorrhea, bruises, chest aliments, circulation, dysentery, gynecopathy, pruritus, rheumatism, sclerosis of the liver, sores and trauma (Blascakova and Poracova, 2011). The aim of the present study was to investigate the potential of an aqueous extract of barley, black plum, and Chinese tree in controlling serum glucose, cholesterol, urea, uric acid and body weight level in alloxan induced diabetic male rabbits compared to normal control and diabetic control male rabbits.

MATERIALS AND METHODS

Animals

Male rabbits were taken from animal house of Institute of Biotechnology and Genetic Engineering University of Sindh Jamshoro and maintained on a normal diet with filtered tap water. The average weight was observed to be 1.4 ± 0.15 kg. The animals were kept for 7-days of acclimatization period.

Experimental design

The rabbits were divided into five groups of 3: (1) alloxan control, (2) normal control and (3-5) diabetic control treated through water extract of barley, black plum and Chinese tree extracts. Diabetes was induced in the rabbits with alloxan monohydrate of 150 mg/dL body weight except normal and control. In the next step, rabbits were screened for diabetes. Rabbits having blood glucose level above 250 mg/dL (in fasting condition) were chosen for the study (as diabetes rabbits). The experiment continued for 21 days. Throughout the period of the treatment all tests were performed, and the body weight was also determined.

Numerical analysis

The data were calculated as mean values and the final value minus initial value, and then divided by the initial value. The answer was multiplied by 100%. The results were compared with control.

Chemicals

Glucose, urea, and cholesterol (Globe diagnostic Itly-GDO34000-GA434000) kits were used for performing the above mentioned tests. Alloxan monohydrate was purchased from Sigma chemical company, U.S. The other chemicals used were of analytical grade and purchased from Sigma, E. Merck and Fluka.

Plant materials

The fresh barley, black plum seeds, and Chinese tree leaves were purchased from local seeds dealers in Hyderabad, Sindh, Pakistan. They were identified by the experts of Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro, Pakistan.

Preparation of plant extracts

Healthy seeds of black plum and barley were selected and kept for 5 min in ethanol and they were dried on filter paper. To prepare water extract, 1 g of barley, black plum seeds, and Chinese tree leaves powder was grinded separately in distilled water. Contents were mixed with glass rod; final volume was made up to 100 mL with distilled water. Clear solution was separated through centrifugation at 5000 rpm for 10 min and samples were stored for further study. Daily dose was used after mixing on magnetic stirrer for 5 min.

Induction of diabetes

Induction of diabetes, alloxan monohydrate was prepared by dissolving 10 g in 100 mL sterilized water. Diabetes was induced in fasting rabbits by administration of 150 mg/kg body weight alloxan to each rabbit.

Administration of extract

100 mL of barley, black plum, and Chinese tree extracts was poured into separate drinking feeders in the morning and then the animals were given free access to water after finishing their extract. 50 mL of extracts was again given orally per rabbit once in the afternoon.

Blood sample collection

Blood samples were collected from marginal vein of the posterior side of the ear of the animals through sterilized syringe and needle. Then the samples were transferred to sterilized micro centrifuge tube for biochemical analysis (serum glucose, serum cholesterol, serum urea, serum uric acid and body weight).

RESULTS AND DISCUSSION

Diabetes mellitus is the most common endocrine metabolic disorder that affects patients' health (Nadir Ali et al., 2013). It occurs due to excess amount of glucose present in blood reacting with haemoglobin to form glycosylated haemoglobin. Glycation rate is directly proportional to the amount of blood glucose (Sheela and Augusti, 1992). There is a proof that in diabetic condition, glycation induces the formation of oxygen-derived free radicals (Gupta et al., 1997).

Plants are used for the treatment of diabetes because they contain several compounds that could act as anti-diabetic agents. These could preserve β-cell function and avoid diabetes (Valsta et al., 2005). Antidiabetic herbal medicines are suggested and given worldwide due to their less side effects and cheaper cost (Shukia et al., 2000). Leaves of *Cydonia oblonga* were used for the treatment of cold, cough, bronchitis, diarrhoea, nervousness and against hyperglycemia (Tabata et al., 1988; Ermis, 2012; Sezik et al., 2001; Tuzlacı and Tolon, 2000). Alloxan behaves as a cytotoxin for β-cells of the islet Langerhans, cause diabetes and induce cell necrosis (Jorns et al., 1997). Intracellular accumulation of alloxan in rabbits leads to β-cell membrane disruption (Mathew and Augusti, 1973). Recently, several researchers have evaluated many traditionally important medicinal plants such as onion, bitter melon, ginger etc that can act as antidiabetic agents (Ramkumar et al., 2007; Pari and Rajarajeswari, 2010).

In alloxan induced diabetic rabbits, the blood glucose levels raised because of permanent destruction of pancreatic β-cell resulting in the reduction of serum insulin level (Hala et al., 2006). It was also reported in Khushk et al. (2010) soya bean extract showed lower blood glucose level as compared to the diabetic control group; therefore it shows that not only barley, black plum, and Chinese tree have antidiabetic effect but some other plants also lower blood glucose level. The rate of reduction in serum glucose level was most effective. The results of barley, black plum, and Chinese tree were obtained by performing blood serum analysis for glucose, cholesterol, urea, uric acid, and body weight. Results are shown in Figures 1 to 5.

The effect of different medicinal plant extract on diabetic male rabbits was observed. Serum glucose level was reduced from 321 to 150 mg/dL when 1.0% barley extract was used. Black plum blood reduced glucose from 297 to 181 mg/dL and Chinese tree serum decreased glucose from 311 mg/dL to 210 mg/dL as shown in Figure 1a to c. According to Virdi et al. (2003), the water extract of fresh unripe whole fruits at a dose of 20 mg/kg was found to trim down fasting glucose level by 48%. The hypoglycemic effect of barley might be due to the presence of β-glycan (Sullivan et al., 2013) and hypoglycemic effect of black plum could be due to the presence of gallic acid.

Alloxan induced rabbits were treated with the water extract of barley and it was observed that serum cholesterol level decreased from 146 to 90 mg/dL; Chinese tree reduced serum cholesterol level from 137 to 120 mg/dL. Black plum cholesterol increased from 136 to 290 mg/dL as reported in another medicinal plant which shows that ginger stimulated the conversion of cholesterol to bile acids, an important path way of eliminating cholesterol from the body and reducing cholesterol level (Nadir Ali et al., 2013). Another study

a   b

c
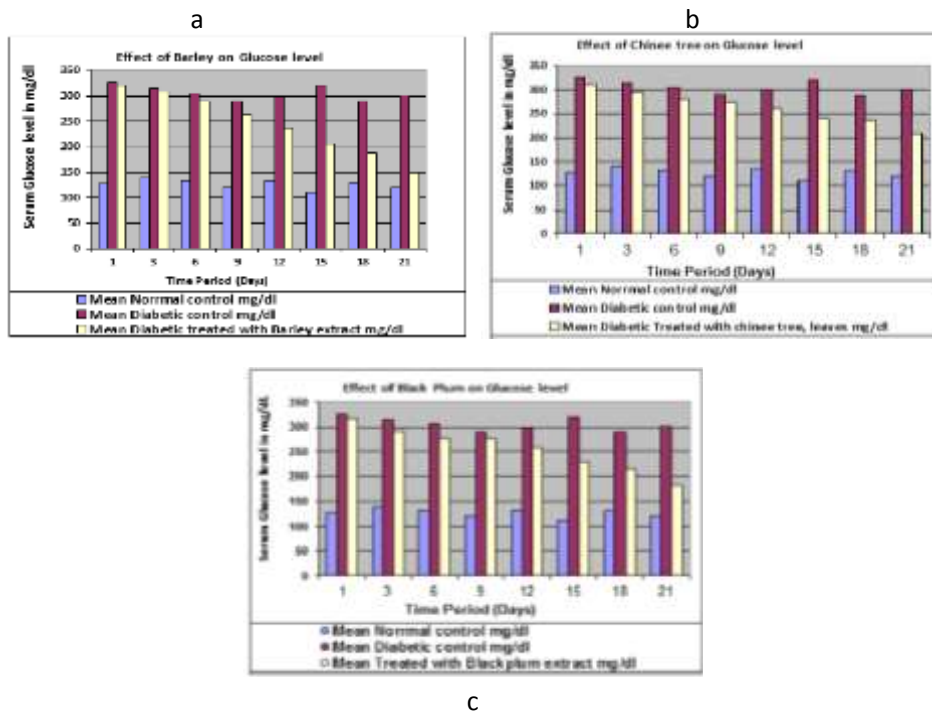
Figure 1. Effect of plants extracts on glucose level in alloxan induced rabbits. (a) Barley extract; (b) Chinese tree leaves extract; and (c) Black plum extract.

a   b

c
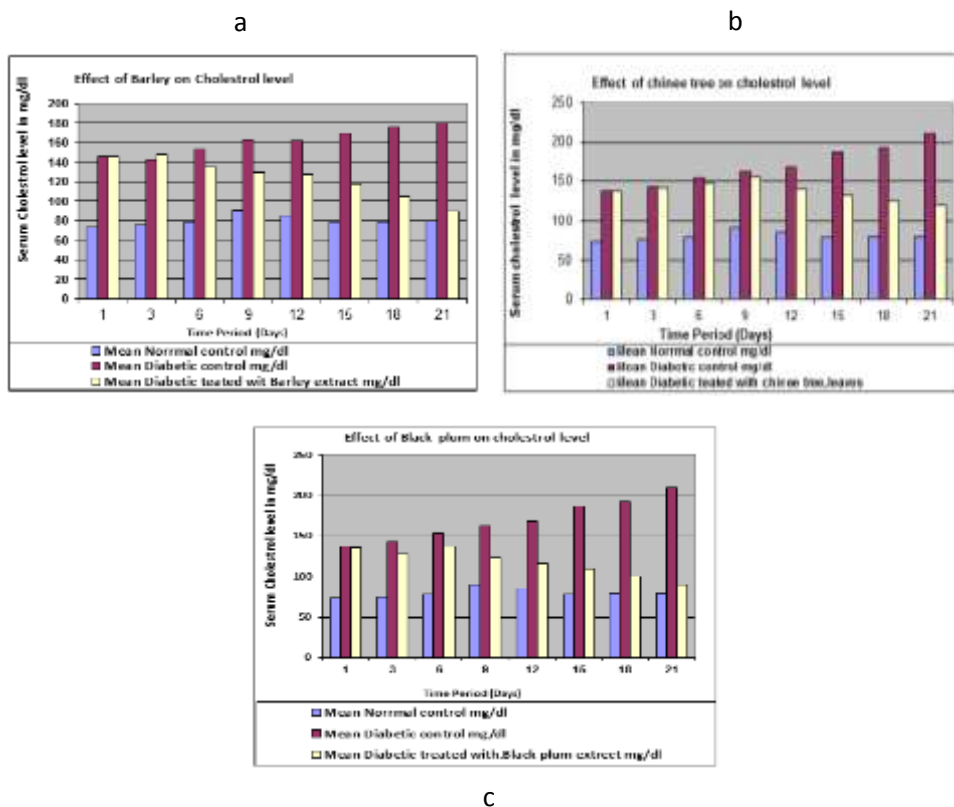
Figure 2. Effect of medicinal plants on cholesterol level in alloxan-induced rabbits; (a) Effect of barley extract; (b) Effect of Chinese tree extract, and (c) Effect of black plum extract.
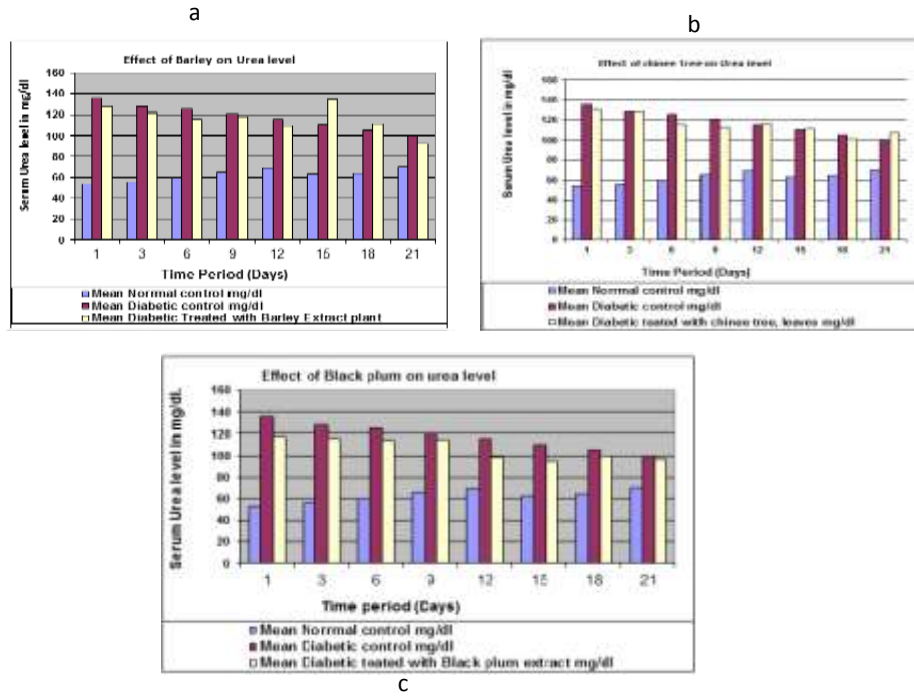
Figure 3. Effect of medicinal plants on urea level in alloxan-induced rabbits; (a) Effect of barley extracts; (b) Effect of Chinese tree extract, and (c) Effect of black plum extract.
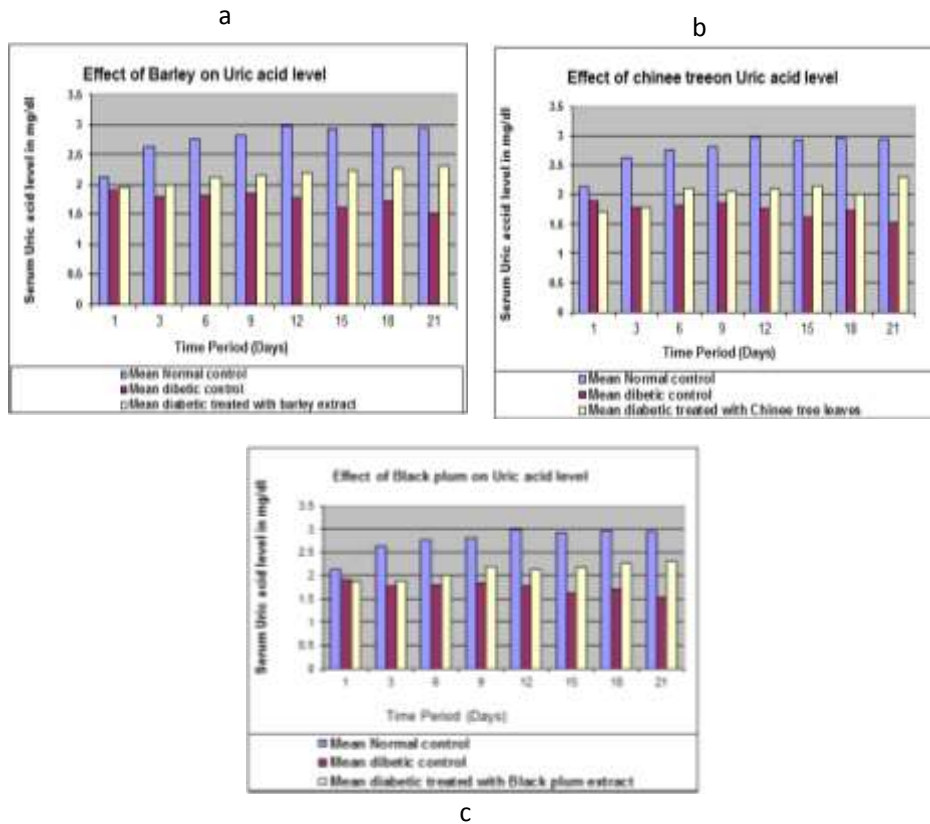
Figure 4. Effect of medicinal plants on serum uric acid in alloxan induced male rabbits. (a) Effect of Barley; (b) Effect of Chinese tree extract; and (c) Effect of black plum extract.
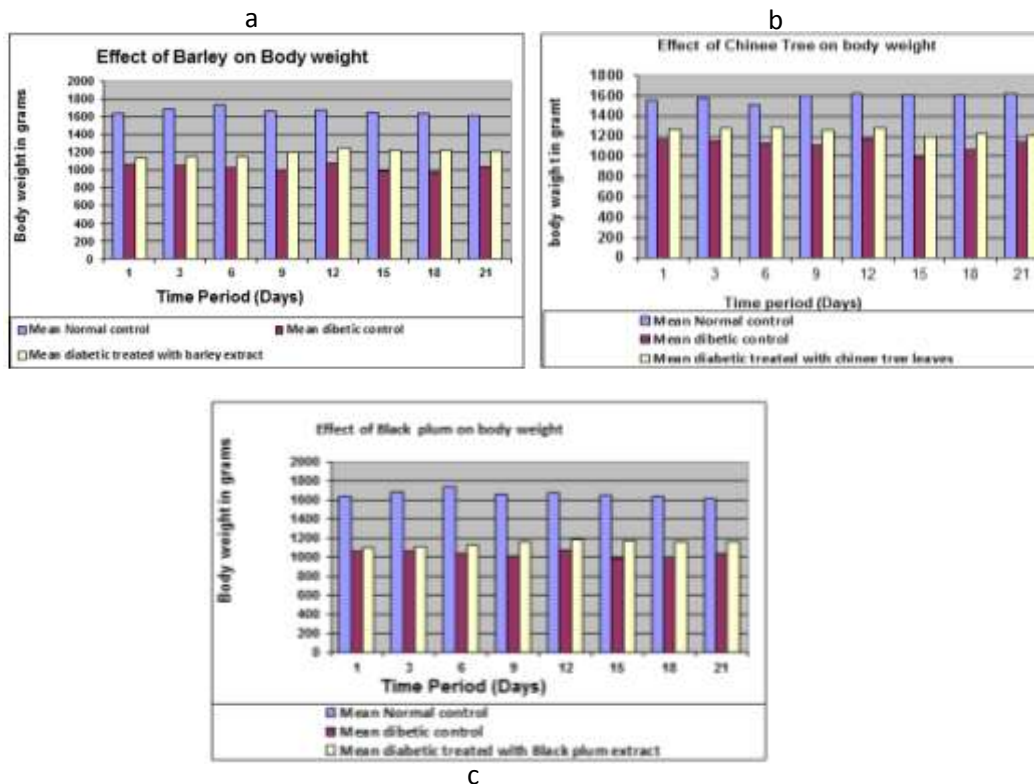
Figure 5. Effect of medicinal plant aqueous extract on body weight of alloxan induced male rabbits. (a) Effect of barley extract; (b) Effect of Chinese tree extract; and (c) Effect of black plum extract.

suggests garlic as a potential antilipidemic, antihypertensive, ant antiglycemic, antithrombotic and antiatherogenic agents which decrease serum triglycerides and cholesterol in diabetic rats (Nadir Ali et al., 2013). The plants with hypoglycemic constituents may have hypolipidemic effects as well. Figure 3 shows the effect of medicinal plants on serum urea level of alloxan induced rabbits. Serum urea level gradually reduced in barley from 127 to 92.31 mg/dL; in black plum it slightly increased from 135 to 137 mg/dL and in Chinese tree leaves, it decreased from 125 to 110.12 mg/dL on the 21st day of the treatment (Figure 3). Untreated diabetic control rabbits reduced the level of serum urea from 135 to 120 mg/dL and Chinese tree, from 135 to 125 mg/dL. Plant extracts were applied on different rabbits group, and slight increase in serum urea level was observed. This might be due to the effect of extract on renal function (Ahmed et al., 2005). Alloxan treatment increased the serum enzymes levels such as cholesterol, LDL, Creatinine, urea and alkaline phosphate and decreased the HDL level, but not glibenclamide (Mohammad et al., 2010). Diabetes cause disturbance in renal function so that the blood urea level is elevated. Extract treated group exhibited reduction in serum urea level, indicating the extract may have effect on renal function as shown in our result (Khushk et al., 2010).

Effect of medicinal plants' aqueous extract on uric acid

level in alloxan-induced rabbits was investigated. The results are depicted in Figure 4. Serum uric acid level increased from 1.96mg/dL to 2.5 mg/dL in barley extract, increased from 1.87 to 2.31 mg/dL in black plum and similar pattern was observed in Chinese tree, from 1.71 to 1.99 mg/dL on the 21st day of treatment as shown in Figure 4. Previous studies have also shown similar effect of plant extract on increasing pattern of uric acid (Mahdi et al., 2003). The results of the effect of medicinal plant aqueous extract on body weight of alloxan induced male rabbits are presented in Figure 5. Body weight increased in rabbits treated with barley from 1140 to 1215 g; in diabetic control, from 1040 to 1065 g; in normal male rabbit, body weight decreased from 1638 to 1615 g. The body weight of rabbits treated with black plum increased from 1100 to 1160 g; in diabetic control it increased from 1040 to 1065 g; in normal male rabbits, it increased from 1638 to 1650 g. While in rabbits treated with Chinese tree their body weight increased from 1230 to 1270 g; in diabetic control, it decreased from 1240 to 1065 g; in normal male rabbit, it decreased from 1638 to 1615 g on the 21st day of the treatment as shown in Figure 5. It shows that the body weight of normal rabbits had greater value compared to diabetic and treated rabbits. In soya bean, it was also reported that the weight gain of the group treated with extract had corrected body metabolism; the results revealed that the soya bean alcohol exhibited

antidiabetic activity in a dose dependent manner (Khushk et al., 2010). The lipolysis, proteolysis, and acute fluid loss during diabetes cause weight loss (Alberti and Zimmet, 1998). Results suggest that oral administration of aqueous extract of barley, black plum and Chinese tree reduced glucose, cholesterol, urea, and uric acid level. Therefore, these medicinal plants should be supplemented as herbal drugs in the treatment of various complex diseases and specially to control diabetes. The active ingredients of anti-diabetes in barley, black plum and Chinese tree leaves should be identified.

Conclusion

This study investigates the effect of medicinal plants (barley, black plum, and Chinese tree leaves) aqueous extract as anti-diabetic agents and their effects on the serum cholesterol, urea, and uric acid and body weight of alloxan induced male rabbits. Herbal medicines have an excellent potential to reduce diabetes in alloxan induced male rabbits. Barley greatly controls diabetes in alloxan induced diabetic male rabbits compared to black plum and Chinese tree. Barley extract reduced diabetes level to 43.92%, black plum to 39.5%, and Chinese tree to 32.47%. Therefore, these medicinal plants should be supplemented as herbal drugs in the treatment of various complex diseases and specially to control diabetes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Ahmed S, Awal M, Rahman M, Mostofa M (2005). Comparative efficacy of neem and karela with insulin and glibenclamide on biochemical parameters in rabbit. Journal of Animal and Veterinary Advances 4(1):103-106.

Alberti KGMM, Zimmet PF (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. Diabetic Medicine 15(7):539-553.

Alu'datt MH, Rababah T, Ereifej K, Alli I, Alrababah MA, Almajwal A, Alhamad MN (2012). Effects of barley flour and barley protein isolate on chemical, functional, nutritional and biological properties of Pita bread. Food Hydrocolloids 26(1):135-143.

Aparicio, MI, AR Cuenca, MJV Suarez and MAZ Revilla (2008). Soybean, a promising health source. Nutricion Hospitalaria 23:305-312.

Behall KM, Scholfield DJ, Hallfrisch J (2006). Whole-grain diets reduce blood pressure in mildly hypercholesterolemic men and women. Journal of The American Dietetic Association 106(9):1445-1449.

Chisholm DJ, Campbell LV, Kraegen EW (1997). Pathogenesis of the insulin resistance syndrome (syndrome X). Clinical and Experimental Pharmacology and Physiology 24(9-10):782-784.

Dans AM, Villarruz MVC, Jimeno CA, Javelosa MAU, Chua J, Bautista R, Velez GGB (2007). The effect of Momordica charantia capsule preparation on glycemic control in type 2 diabetes mellitus needs further studies. Journal of Clinical Epidemiology 60(6):554-559.

Dongowski G, Huth M, Gebhardt E, Flamme W (2002). Dietary fiber-rich barley products beneficially affect the intestinal tract of rats. Journal of Nutrition 132(12):3704-3714.

Ermis A (2012). Isırgan tohumlu bitki karışımının alkole bağlı karaciğer hasarları üzerine etkisinin biyokimyasal ve histokimyasal araştırılması (Master's thesis, Pamukkale Üniversitesi Fen Bilimleri Enstitüsü).

Gupta B, Nehal M, Baquer N (1997). Effect of experimental diabetes on the activities of hexokinase, glucose-6-phosphate dehydrogenase and catecholamines in rat erythrocytes of different ages. Indian. Journal of Experimental Biology 35(7):792-795.

Hala M, Eman M, Aataa A (2006). Antihyperglycemic, antihyperlipidemic and antioxidant effects of Ziziphus spina-christi and Ziziphus jujuba in alloxan induced diabetic rats. International Journal of Pharmacology 2:563-570.

Hoang MH, Houng SJ, Jun HJ, Lee JH, Choi JW, Kim SH, Lee SJ (2011). Barley intake induces bile acid excretion by reduced expression of intestinal ASBT and NPC1L1 in C57BL/6J mice. Journal of Agricultural and Food Chemistry 59(12):6798-6805.

Hosseini A, Shafiee-Nick R, Ghorbani A (2015). Pancreatic beta cell protection/regeneration with phytotherapy. Brazilian Journal of Pharmaceutical Sciences 51(1):1-16.

Hussain Mir S, Maqbool Darzi M, Saleem Mir M (2013). Efficacy of Abroma augusta on biochemical and histomorphological features of Alloxan-induced diabetic rabbits. Iranian Journal of Pathology 8(3):153-158.

Jain R, Vyas C (1974). Hypoglycaemia action of onion on rabbits. British Medical Journal 2(5921):730.

Jorns A, Munday R, Tiedge M, Lenzen S (1997). Comparative toxicity of alloxan, N-alkylalloxans and ninhydrin to isolated pancreatic islets *in vitro*. Journal of Endocrinology 155(2):283-293.

Jousilahti P, Sarwar N, Gao P, Seshasai S, Gobin R, Kaptoge S, Selvin E (2010). Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. Lancet 375(9733):2215-22.

Kanauchi O, Fujiyama Y, Mitsuyama K, Araki Y, Ishii T, Nakamura T, Andoh A (1999). Increased growth of Bifidobacterium and Eubacterium by germinated barley foodstuff, accompanied by enhanced butyrate production in healthy volunteers. International Journal of Molecular Medicine 3(2):175-184.

Kemp LP, Paik WC, Scheid MP (2013). Natural Nutritional Supplements: No. 13/451,329.

Khushk I, Dahot MU, Baloach SA, Bhutto MA (2010). The evaluation of soybean extracts in alloxan induced diabetic rabbits. World Applied Sciences Journal 8:22-25.

Wadood N, Nisar M, Rashid A, Wadood A, Gul-Nawab, Khan A. (2007). Effect of a compound recipe (medicinal plants) on serum insulin levels of alloxan induced diabetic rabbits. Journal of Ayub Medical College Abbottabad 19(1):32-38

Kumar S, Kumar V, Prakash O (2011). Antidiabetic, hypolipidemic and histopathological analysis of Dillenia indica (L.) leaves extract on alloxan induced diabetic rats. Asian Pacific Journal of Tropical Medicine 4(5):347-352.

Lillioja S, Mott DM, Spraul M, Ferraro R, Foley JE, Ravussin E., Bogardus C (1993). Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus: prospective studies of Pima Indians. The New England Journal of Medicine 329(27):1988-1992.

Mahesar H, Bhutto MA, Khand AA, Narejo NT (2010).Garlic used as an alternative medicine to control diabetic mellitus in alloxan induced male rabbits. Pakistan Journal of Physiology6(1):39-41

Mahdi AA, Chandra A, Singh RK, Shukla S, Mishra L, Ahmad S (2003). Effect of herbal hypoglycemic agents on oxidative stress and antioxidant status in diabetic rats. Indian Journal of Clinical Biochemistry 18(2):8-15.

Mathew P, Augusti K (1973). Studies on the effect of allicin (diallyl disulphide-oxide) on alloxan diabetes. I. Hypoglycaemic action and enhancement of serum insulin effect and glycogen synthesis. Indian Journal of Biochemistry and Biophysics 10(3):209-212.

Mendoza- Lopez ML, Alvarado Diaz CS, Perez Vega SB., Leal Ramos MY, Gutierrez Mendez N (2017). Compositional and free radical scavenging properties of Zea mays female inflorescences (maize silks) from Mexican maize landraces. CyTA-Journal of Food 16(1):96-104.

Muhammad , FA, S M kazim, S S Ghor, SS Mehjabeen, RA Sheikh, MA Sheikh , M Ibrahim (2010). Antidiabetic Activity of Vinca rosea extracts in alloxan-induced diabetic rats. International Journal of Endocrinology Article ID 841090, 6.

Mutalik S, Sulochana B, Chetana M, Udupa N, Devi, PU (2003). Preliminary studies on acute and subacute toxicity of an antidiabetic herbal preparation, Dianex. Indian Journal of Experimental Biology 41(4):316-320.

Nadir Ali Rind, Umar Dahot, Malik SA, Kumar M, Bhutto MA, Rafiq M (2013). Comparative antihyperglycemic acitivity of aqueous extracts of garlic (Allium sativum) and ginger (Zingiber afficinale) in alloxan induced male rabbits. Pakistan Journal of Biotechnology 10(2):53-62

Pari L, Rajarajeswari N (2010). Protective role of coumarin on plasma and tissue glycoprotein components in streptozotocin-nicotinamide induced hyperglycemic rats. International Journal of Biological and Medical Research 1(3):61-65.

Rahelic D (2016). 7th edition of idf diabetes atlas-call for immediate action. Liječnički vjesnik 138(1-2):57-58.

Ramkumar KM, Rajaguru P, Latha M, Ananthan R (2007). Ethanol extract of Gymnema montanum leaves reduces glycoprotein components in experimental diabetes. Nutrition Research 27(2):97-103.

Sezik E, Yesilada E, Honda G, Takaishi Y, Takeda Y, Tanaka T (2001). Traditional medicine in Turkey X. Folk medicine in central Anatolia. Journal of Ethnopharmacology 75(2):95-115.

Sharma B, Balomajumder C, Roy P (2008). Hypoglycemic and hypolipidemic effects of flavonoid rich extract from Eugenia jambolana seeds on streptozotocin induced diabetic rats. Food and Chemical Toxicology 46(7):2376-2383.

Sheela C, Augusti K (1992). Antidiabetic effects of S-allyl cysteine sulphoxide isolated from garlic Allium sativum Linn. Indian Journal of Experimental Biology 30(6):523-526.

Shukia R, Sharma S, Puri D, Prabhu K, Murthy, P (2000). Medicinal plants for treatment of diabetes mellitus. Food and Chemical Toxicology 15(1):169-177.

Sullivan P, Arendt E, Gallagher E (2013). The increasing use of barley and barley by-products in the production of healthier baked goods. Trends in Food Science and Technology 29(2):124-134.

Tabata M, Honda G, Sezik E (1988). A report on traditional medicine and medicinal plants in Turkey. Faculty of Pharmaceutical Sciences, Kyoto University.

Tada R, Ikeda F, Aoki K, Yoshikawa M, Kato Y, Adachi Y, Ohno N (2009). Barley-derived β-D-glucan induces immunostimulation via a dectin-1-mediated pathway. Immunology Letters 123(2):144-148.

Thomson I. (2006). TP Prohp, IO Onoagbe, PC Onyebuagu, AA Omeni, RI Okoli and NP Obeto. Pakistan Journal of Nutrition 5(5):425-428.

Tuzlacı E, Tolon E (2000). Turkish folk medicinal plants, part III: Sile (Istanbul). Fitoterapia 71(6):673-685.

Valsta L, Tapanainen H, Mannisto S (2005). Meat fats in nutrition. Meat Science 70(3):525-530.

Virdi J, Sivakami S, Shahani S, Suthar A, Banavalikar M, Biyani M (2003). Antihyperglycemic effects of three extracts from Momordica charantia. Journal of Ethnopharmacology 88(1):107-111.

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Genetic diversity in *Radix* species from the middle and south of Iraq based on simple sequence repeats

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Ten newly developed microsatellite loci were isolated from an AG_{20} and CAG_{20} enriched genomic DNA library of *Radix* sp. from Iraq. The simple sequence repeats (SSR) comprised 76.0% di-, 20.0% tri- and 4.0% tetra-nucleotide repeats. The number of alleles for all 10 loci ranged from 4 to 18, with a mean of 11.3 alleles per locus. The observed (Ho) and expected heterozygosity (He) values varied from 0.286 to 0.927 (mean 0.640) and from 0.263 to 0.939 (mean 0.753), respectively. The polymorphic information content (PIC) values ranged from 0.336 to 0.923 (mean 0.749). Five loci showed significant deviation from Hardy-Weinberg equilibrium (HWE) and no significant linkage disequilibrium (LD) was observed. Cross transferability was successfully tested on species from the genus *Melanopsis*. These polymorphic SSRs will be useful for assessment of the genetic diversity and population genetics of a *Radix* sp. as well as other related species in Iraq.

Key words: Cross-transferability, enriched genomic DNA library, freshwater snail, microsatellite.

INTRODUCTION

The genus *Radix* Montfort, 1810 is a genus of freshwater snails belonging to the family of Lymnaeidae (Gastropoda: Pulmonata) which consists of 1800 species and 34 genera (Hubendick, 1951; Vinarski, 2013). Species identification of this family is classically based on morphological characteristics of the shell (Jackiewicz, 2000), reproductive organs (Hubendick, 1951) and mantle pigmentation (Jackiewicz, 1998), although more

recently, identification has included molecular characterization (Pfenninger et al., 2006; Dung et al., 2013). Species in the genus *Radix* are vectors of human parasites such as *Trichobilharzia szidati*, *Trichobilharzia franki* and *Fasciola hepatica* (Ferté et al., 2005; Caron et al., 2007).

Radix sp. samples from the middle and south of Iraq show highly polymorphic shell, mantle pigmentation

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patterns and mating systems (Al-Waaly et al., 2014). Although Glöer and Pešić (2012) considered *R. auriculaia* to be native to Iraq, and Seddon et al. (2014) mentioned that the specimens from the Southwest Asia are currently assigned to *R. auricularia*, Naser et al. (2008) found that there was no real presence of this species in the middle and south regions of Iraq. However, there is no information of the genetic diversity of *Radix* sp. in the middle and south of Iraq. Thus, in order to obtain a better understanding of this species, its genetic diversity needs to be evaluated.

Microsatellites or SSRs (simple sequence repeats) are genetic markers that are useful for the assessment of genetic diversity due to their abundance, random distribution in the genome, high polymorphism, co-dominance, multi-allelic nature, reproducibility and high transferability (Powell et al., 1996; Gupta and Varshney, 2000). Cross-transferability of SSRs has been successfully applied to study within or between Taxa (BarbarÁ et al., 2007). There were many reports of successful cross-amplification between species/within genus such as scallop (Ibarra et al., 2006), sea urchin (Carlon and LippÉ, 2007) and fish (VyskoČIlovÁ et al., 2007), between genera/within family such as crayfish (Belfiore and May, 2000), bird (Chbel et al., 2002) and tubeworm (McMullin et al., 2004), between families/within order such as horseshoe bat (Dawson et al., 2004), flatfish (Iyengar et al., 2000) and deer (Gaur et al., 2003). In addition, SSRs have been developed and studied in members of the lymnaeid genera, namely; *Lymnaea truncatula* (Trouve et al., 2000), *Lymnaea stagnails* (Knott et al., 2003), *Lymnaea auricularia* (de Boer et al., 2004) and *Radix balthica* (Salinger and Pfenninger, 2009). In freshwater snails, SSRs were successfully cross-amplified between species/within genus (Trouve et al., 2000; Gow et al., 2001; Wilmer et al., 2005; Dubois et al., 2008; Liu and Hershler, 2008; Dupuy et al., 2009; Nicot et al., 2009; Salinger and Pfenninger, 2009; Gu et al., 2012a, 2012b, 2015) and between genera/within family (Wilmer et al., 2005).

Melanopsis spp. is a freshwater snail, belonging to the family Melanopsidae (Gastropoda: Caenogastropoda: Cerithioidea). This genus is also widely distributed in Iraq (Naser, 2006; Al-Waaly et al., 2014; Mohammad et al., 2014). However, no information on SSR marker and genetic diversity of *Melanopsis* spp. are available. Although no cross-transferability between families/within order of freshwater snail has been reported, successful transferability of SSR marker has been demonstrated in many studies (Iyengar et al., 2000; Gaur et al., 2003; Dawson et al., 2004). In addition, SSRs have also been widely used in population genetics and phylogenetic studies in insects (Daly-Engel et al., 2012), birds (Parine et al., 2013), fish (Nugraha et al., 2014) and snails (Stoeckle et al., 2014). This study therefore aimed to develop species specific SSR markers for genetic diversity study of rom Radix sp. from the middle and south of Iraq

and to apply these newly developed SSRs in *Melanopsis* spp.

MATERIALS AND METHODS

Experimental animals

A total of 30 *Radix* sp. were collected using a mesh scoop with a wire net of 16 meshes per inch from the middle and south provinces of Iraq, located at Khoura (N 30° 36` 36`, E 47° 46` 12`) and Garmat Ali (N 30° 39` 40`, E 47° 31` 50`) in Basra, Al-Kahlaa (N 31° 40` 35`, E 47° 16` 43`) in Missan, Ukhaidher (N 32° 27` 42`, E 43° 35` 42`) in Karbala, Al-Hussainiya (N 32° 25` 56`, E 45° 52` 30`) in Wasit, Al-Wardia (N 32° 29` 58`, E 44° 27` 23`) in Babil, Al-Rumaitha (N 31° 31` 50`, E 45° 12` 19`) in Muthanna and Al-Sanniya (N 32° 03` 55`, E 44° 46` 24`) in Al-Qadisiyah. Cross transferability analysis was evaluated using three freshwater snail samples each of species of the *Melanopsis* genus, namely *Melanopsis costata* from Daghara (N 32° 08` 13`, E 44° 55` 53`) in Al-Qadisiyah, *Melanopsis nodosa* from Chebayish (N 30° 57` 20`, E 46° 58` 35`) in Dhi Qar and *Melanopsis buccinoidea* from Ain Tamur (N 32° 34` 35`, E 43° 30` 34`) in Karbala (Figure 1 and Table 1). *Radix* sp. and *Melanopsis* spp. samples were preserved in 70% ethanol and genomic DNA was extracted from foot tissue using a Genomic DNA Extraction kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturers' instructions.

SSR-enriched library

Isolation of microsatellite loci from a *Radix* sp. (AG_{20} and CAG_{20}) enriched library was performed as described by Sraphet et al. (2011) with some modifications. Genomic DNA of *Radix* sp. samples was extracted and digested with *Alu*I (Takara, Japan), *Hae*III (Takara, Japan) and *Afa*I (Takara, Japan) restriction enzymes, then purified using the Wizard SV gel and PCR Clean-Up system (Promega, Madison, WI, USA). Purified DNA of each digestion reaction was ligated with linkers and hybridized with biotinylated oligonucleotide probes and subsequently captured using streptavidin-coated magnetic beads (Invitrogen Dynal AS, Norway). Amplified DNA fragments in a size range of 0.5 - 1.0 kbp were selected to clone into the pGEM®-T Easy Vector System (Promega). Recombinant plasmids were transformed into DH5α-competent *Escherichia coli* cells and plated on LB-agar containing 100 μg/ml ampicillin, 20 mM IPTG and 80 μg/ml of X-gal. Positive colonies containing the expected insert sizes were selected by PCR amplification with vector primers and sequenced. SSR motifs were found using the Web-Sat program (http://wsmartins.net/websat/) (Martins et al., 2009) and redundant sequences were checked using the CAP3 program (http://doua.prabi.fr/software/cap3) (Huang and Madan, 1999). SSR primers were designed using the Web-Sat program.

SSR analysis

Genomic DNA of 30 *Radix* sp. and three individual samples of each *M. costata, M. nodosa* and *M. buccinoidea* were tested with SSR primers. PCR reactions were performed as described by Sraphet et al. (2015) with some modifications, in a total volume of 20 μl containing 15 ng of DNA, 1× *Taq* buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 2 μM of each primer and 1 U of *Taq* DNA polymerase (Thermo Scientific, Foster City, CA). PCR reactions were carried out with denaturation at 94ºC for 2 min, followed by 35 cycles of amplification at 94°C for 30 s, 55°C for 45 s and 72°C for 1 min with a final extension at 72°C for 5 min. The amplified products were

Figure 1. The map of Iraq showing collection sites of *Radix* sp. (● *Melanopsis nodosa* (◆ , *M. costata* (▲) and *M. buccinoidea* (■).

analyzed on 5% polyacrylamide gels and visualized by silver staining (Benbouza et al., 2006).

Data analysis

The observed heterozygosity (Ho), expected heterozygosity (He), deviations from Hardy-Weinberg equilibrium (HWE) for each locus and polymorphic information content (PIC) were calculated using genotypic data by PowerMarker V3.25 (Liu and Muse, 2005). The linkage disequilibrium (LD) between all loci was tested using the online version of GENEPOP on the web (Raymond and Rousset,

1995). Cluster analysis and construction of the dendrogram were performed by the Un-weighted Pair-Group Method (UPGMA) using the TFPGA program (Miller, 1997).

RESULTS

Microsatellite characterization

A total of 136 positive clones from a *Radix* sp. (AG$_{20}$ and CAG$_{20}$) enriched library were selected for DNA

Table 1. Description of *Radix* sp. and *Melanopsis* spp. collection sites from the middle and south of Iraq.

Species	Collection site	Latitude and longitude	Code
Radix sp.	Basra, Khoura	N 30° 36` 36`, E 47° 46` 12`	4, 5, 6, 13, 24
	Basra, Garmat Ali	N 30° 39` 40`, E 47° 31` 50`	1, 2, 3, 15, 23
	Missan, Al-Kahlaa	N 31° 40` 35`, E 47° 16` 43`	7, 8, 9, 18
	Karbala, Ukhaidher	N 32° 27` 42`, E 43° 35` 42`	10, 11, 12
	Wasit, Al-Hussainiya	N 32° 25` 56`, E 45° 52` 30`	16, 19, 20, 25, 26, 27
	Babil, Al-Wardia	N 32° 29` 58`, E 44° 27` 23`	14, 17
	Muthanna, Al-Rumaitha	N 31° 31` 50`, E 45° 12` 19`	21, 22
	Al-Qadisyah, Al-Sanniya	N 32° 03` 55`, E 44° 46` 24`	28, 29, 30
M. costata	Al-Qadisiyah, Daghara	N 32° 08` 13`, E 44° 55` 53`	31, 32, 33
M. nodosa	Dhi Qar, Chebayish	N 30° 57` 20`, E 46° 58` 35`	34, 35, 36
M. buccinoidea	Karbala, Ain Tamur	N 32° 34` 35`, E 43° 30` 34`	37, 38, 39

Table 2. Type and number of microsatellite motifs from *Radix* sp.

Type of SSR motif	No. of clone (%)	Repeat motif	No. of clone (%)
Di-nucleotide	38 (76.0)	AG/TC	30 (60.0)
		CA/GT	8 (16.0)
Tri-nucleotide	10 (20.0)	CAG/GTC	8 (16.0)
		CAA/GTT	2 (4.0)
Tetra-nucleotide	2 (4.0)	AAAC/TTTG	1 (2.0)
		ATGC/TACG	1 (2.0)

sequencing. Of the 136 clones, 110 (80.9%) were successfully sequenced. Of these, 76 (69.1%) and 34 (30.9%) contained microsatellite motifs with non-redundant and redundant sequences, respectively. Three types of microsatellite motifs were found, consisting of 76.0% (38 sequences) di-, 20.0% (10 sequences) tri- and 4.0% (4 sequences) tetra-nucleotide repeats (Table 2).

Genetic diversity of microsatellite markers

A total of 50 new SSR primer pairs were designed based on the microsatellite sequences. Of these 50 pairs of SSR primers, 10 pairs showed polymorphisms and these were used to analyze 30 samples of *Radix* sp., resulting in total of 113 alleles (Table 3 and Figure 2). The number of alleles per locus ranged from 4 (Ra22) to 18 (Ra2), with an average of 11.3 alleles per locus. The observed heterozygosity (Ho) of these polymorphic loci ranged from 0.286 (Ra34) to 0.927 (Ra26) with an average of 0.640, while expected heterozygosity (He) ranged from 0.263 (Ra34) to 0.939 (Ra2) with an average of 0.753. Significant deviation from Hardy-Weinberg equilibrium were observed in this study at the loci Ra2, Ra18, Ra32,

Ra42 and Ra49 ($p < 0.05$). Tests for linkage disequilibrium ($p < 0.001$) revealed no linkage disequilibrium among the pair-wise compared loci. In addition, the PIC value of polymorphic SSRs ranged between 0.336 (Ra34) and 0.923 (Ra2), with an average of 0.749.

Cross-genera transferability

All 10 polymorphic primers derived from *Radix* sp. were tested with three individuals of each species from the genus *Melanopsis*, namely *M. costata*, *M. nodosa* and *M. buccinoidea* (Table 3). All 10 loci were successfully amplified for at least one species. Of these, four (Ra22, Ra32, Ra34 and Ra42), six (Ra18, Ra22, Ra32, Ra34, Ra37 and Ra42) and four loci (Ra22, Ra32, Ra34 and Ra42) were amplified for three individual samples of *M. costata, M. nodosa* and *M. buccinoidea*, respectively (Table 4).

UPGMA clustering by SSRs

The genetic distance according to Nei (1972) was

Table 3. Locus name, GenBank Accession no., repeat motif, primer sequence, annealing temperature (Ta), size, number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC), significant deviation of P-value for Hardy–Weinberg equilibrium (HWE) and number of alleles cross-taxa amplification of 10 polymorphic loci from *Radix* sp.

| No.	Locus	GenBank Accession no.	Repeat motif	Primer sequence (5'-3')	Ta (°C)	Size (bp)	Ho	He	PIC	HWE (p-value)	*Radix* sp.	*M. costata*	*M. nodosa*	*M. buccinoidea*
1	Ra2	KJ740525	(CA)14	C F:AAATCACGTTTTATCTCAGCG T R:AAACATCCTCTCCGTACCCAC	55	261	0.500	0.939	0.923	0.000*	18	0	0	1
2	Ra18	KJ740526	(TC)10	A F:AAACAATGGCAACTACAACCC C R:GTGGTCTCAGGTAACAAAACA	55	360	0.853	0.655	0.833	0.034*	10	1	2	2
3	Ra20	KJ740527	(TC)9	G F:GCGTGAAATATGTGTTTTGTG A R:GGGAGAGAGAGAAAGAGAGGG	55	213	0.478	0.410	0.547	1.000	6	1	0	0
4	Ra22	KJ740528	(AG)9	T F:GCGAAACAAATGAGAGAGAGA T R:GTTAGCCTTGTAGCAGAAGCTC	55	260	0.733	0.539	0.421	0.052	4	2	2	2
5	Ra26	KJ740529	(TC)23	A F:GTTCAAAATCACCACCCGTAT G R:AGAAAGTGAGAGAGGCAGAGC	55	197	0.927	0.862	0.910	0.133	17	0	3	3
6	Ra32	KJ740530	(AG)17	A F:CCTTGAGGGCGTTACTTGATA T R:CTCTCTCTCTCTCTCTCTCCCC	55	231	0.786	0.896	0.881	0.009*	14	2	2	2
7	Ra34	KJ740531	(CAG)12	C F:AGCAGTCACAACGTCAACAG G R:TGGTGCCATTTAGGAGATGAG	55	182	0.286	0.263	0.336	1.000	5	2	2	2
8	Ra37	KJ740532	(GT)9	T F:TGTGTGGACAAATCCTTCG G R:CCTAAAATCGTGGTTAATGGA	55	320	0.828	0.900	0.882	0.180	14	1	4	3
9	Ra42	KJ740533	(GA)19	T F:CGTCAGTAGCCAATAGGACAA G R:AGTCAGCAAGGTCAAAGGG	55	172	0.867	0.919	0.896	0.002*	15	2	2	2
10	Ra49	KJ740534	(TC)20	A F:ATCCGAGGCTACCCCTAAATA G R:GGCAGAGAGAGAGAGACAGAG	55	298	0.400	0.881	0.865	0.000*	10	0	1	1
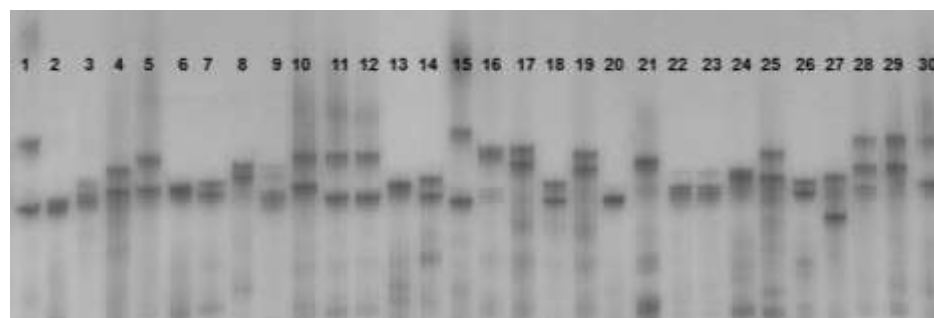
Figure 2. The example of SSR polymorphic pattern (Ra 42) in *Radix* sp.

Table 4. Specific observed allele in *Radix* sp. and genus *Melanopsis*. (* indicates genotype found in specific species tested in cross-transferability of SSRs.).

Individual name		Locus									
		Ra2	Ra18	Ra20	Ra22	Ra26	Ra32	Ra34	Ra37	Ra42	Ra49
Radix sp.	1	2/17	1/10	1/3	2/2	3/7	4/11	1/1	2/10	2/13	2/2
	2	0/0	2/2	0/0	2/3	3/11	7/8	1/5	3/3	12/13	7/7
	3	8/15	4/8	0/0	3/3	10/11	4/5	1/5	6/10	11/12	5/8
	4	14/14	1/5	3/3	2/2	10/13	6/7	1/1	2/7	6/10	6/9
	5	7/7	4/5	3/3	2/3	2/4	3/9	1/5	5/8	5/10	4/6
	6	4/14	2/8	3/3	3/3	4/8	3/8	1/1	4/10	10/10	6/10
	7	1/15	3/9	3/4	2/3	6/11	9/11	0/0	3/11	9/11	1/1
	8	7/7	3/3	3/4	2/3	5/9	6/14	1/1	3/14	6/8	3/8
	9	0/0	0/0	0/0	2/3	1/11	0/0	1/1	0/0	11/12	3/3
	10	5/18	8/9	3/3	2/3	4/16	4/5	1/1	9/13	5/10	0/0
	11	5/5	8/8	3/3	2/3	4/16	2/5	1/2	10/12	10/12	0/0
	12	5/5	5/9	0/0	3/4	3/11	4/6	1/1	6/13	10/12	6/6
	13	13/13	4/7	3/4	2/3	6/10	4/5	1/1	6/9	10/10	0/0
	14	11/11	5/8	2/3	2/3	0/0	6/13	1/1	6/11	9/12	6/8
	15	3/6	4/5	3/3	3/3	4/9	4/5	1/1	5/6	1/13	0/0
	16	16/17	4/7	3/4	2/3	11/11	4/9	1/3	6/10	4/5	7/8
	17	13/13	3/5	3/6	2/3	4/5	7/7	1/1	2/6	4/6	6/9
	18	7/7	5/5	3/3	3/3	9/14	7/12	1/1	5/5	10/14	6/6
	19	2/11	5/6	3/4	1/2	9/10	2/6	1/1	5/6	5/7	1/1
	20	7/17	2/8	3/3	3/3	8/14	9/9	1/1	2/10	13/13	10/10
	21	12/12	2/5	3/3	2/3	3/11	9/9	1/1	2/7	6/6	8/8
	22	6/6	9/9	0/0	2/3	3/6	4/9	1/1	12/12	11/12	6/9
	23	10/17	1/6	0/0	2/3	3/7	3/4	1/1	1/7	11/12	9/9
	24	9/11	5/5	3/3	2/2	14/15	6/10	1/5	5/8	8/9	2/2
	25	17/17	9/9	0/0	2/3	12/17	1/4	1/1	12/13	5/9	2/2
	26	17/18	3/4	3/4	2/3	10/10	0/0	0/0	3/5	10/12	0/0
	27	6/6	5/5	2/3	2/3	2/9	6/11	1/3	6/7	9/15	6/7
	28	4/11	5/5	3/5	2/3	9/9	7/7	1/1	6/6	3/7	5/5
	29	4/4	5/5	3/3	2/3	3/4	7/7	1/1	6/6	3/7	8/8
	30	2/16	4/6	3/3	2/3	12/12	9/9	1/4	4/6	3/10	8/8
M. costata	31	0/0	6/6*	0/0	2/3	0/0	5/7*	5/5*	7/7*	11/12	0/0
	32	0/0	6/6*	3/3	2/2	0/0	5/7*	1/1	7/7*	11/12	0/0
	33	0/0	0/0	0/0	2/3	0/0	5/7*	1/1	0/0	11/12	0/0
M. nodosa	34	0/0	6/6*	0/0	2/3	13/16*	5/7*	1/1	7/11*	11/12	0/0
	35	0/0	5/6	0/0	2/2	12/12	5/7*	1/1	5/6	11/12	8/8
	36	0/0	6/6*	0/0	2/2	0/0	5/5*	1/5	7/7*	11/12	0/0
M. buccinoidea	37	2/2*	5/5	0/0	3/3	9/12*	5/8*	1/1	6/6	11/12	8/8
	38	0/0	2/5	0/0	2/2	13/13*	5/5*	1/6*	7/8*	11/12	0/0
	39	0/0	0/0	0/0	2/2	0/0	5/5*	1/1	0/0	11/12	0/0

calculated amongst individual sample of *Radix* sp. and *Melanopsis* spp. UPGMA clustering based on Nei's method showed that most of *Melanopsis* spp. was clustered together and clearly separated from *Radix* sp. except *M. buccinoidea* (No. 37) as shown in Figure 3. *Radix* sp. (No. 9) was included with *Melanopsis* spp. cluster. Among the group of *Radix* sp. samples and the *Melanopsis* genus, UPGMA clustering was divided into 2
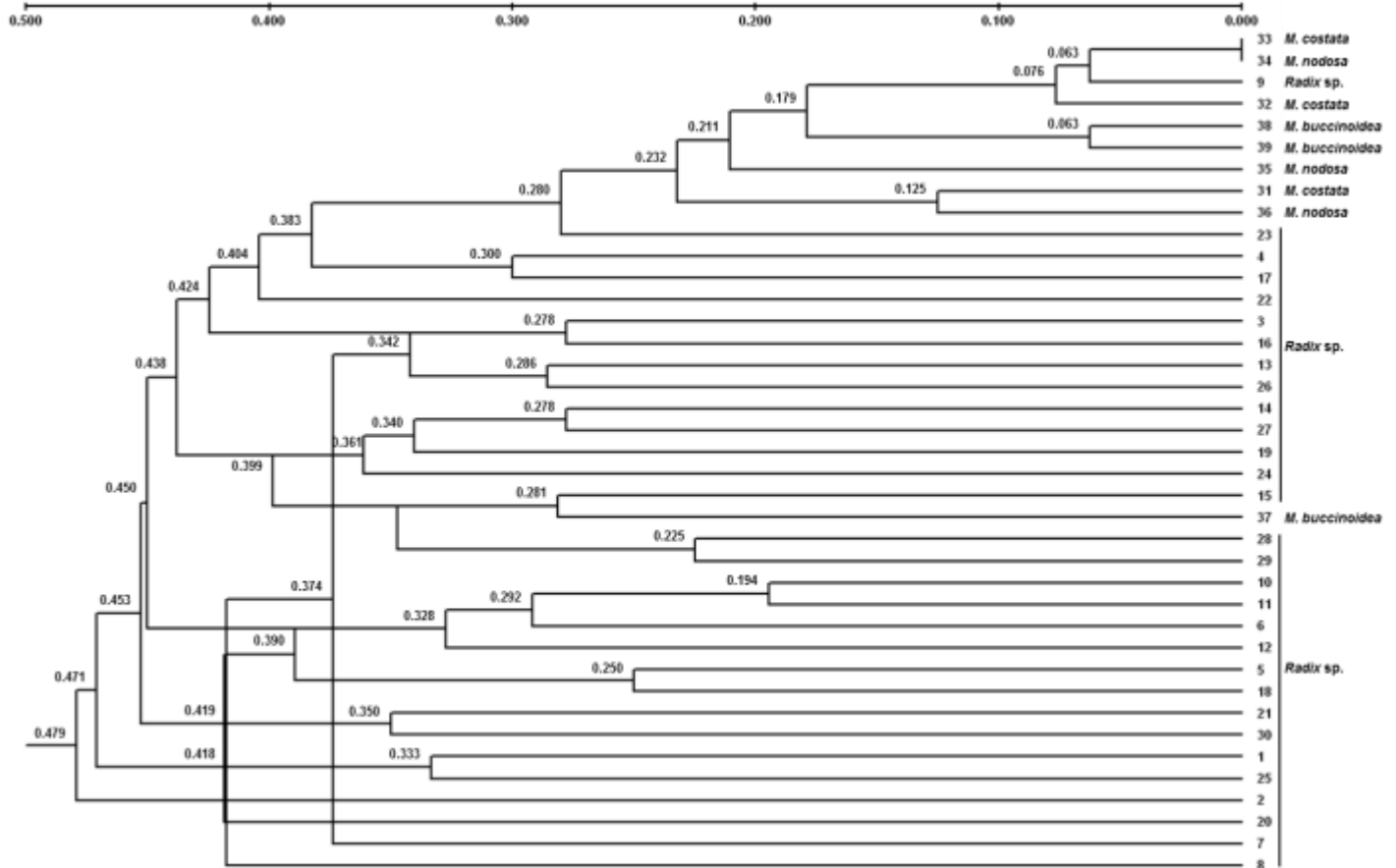
Figure 3. The UPGMA dendrogram based on Nei's genetic distance showing the genetic distance between *Radix* sp. and *Melanopsis* spp. from the middle and south provinces of Iraq.

groups consisting of genus *Melanopsis* (*M. costata*, *M. nodosa*, and *M. buccinoidea*) and *Radix* sp. (Fig 4). The smallest genetic distance (0.073) was observed between *M. costata* and *M. nodosa* while the highest genetic distance (0.518) was observed between *M. costata* and *M. buccinoidea* (Table 5).

DISCUSSION

The SSR-enriched library from *Radix* sp. showed high efficiency due to the high percentage of positive clones containing insert fragments with the expected size and a reasonable percentage of non-redundancy. While enrichment increased the number of positive clones which contained microsatellite motifs, the level of clone redundancy was still quite high, albeit similar to the redundancy rate found in the study of Zane et al. (2002). The polymorphism rate of SSR primers developed from *Radix* sp. enriched library was slightly lower than other species of freshwater snail genus such as *Physa acuta* (Monsutti and Perrin, 1999), *Aplexa marmorata* (Dubois et al., 2008) and *Bellamya aeruginosa* (Gu et al., 2015).

This may be due to the nature of genetic background as well as differences in genetic diversity of this species from each location.

In this study, the number of alleles at each locus was higher than previous reports of *R. balthica* (Salinger and Pfenninger, 2009) and also other freshwater snail libraries such as for *L. stagnalis* (Knott et al., 2003), *Physa marmorata* (Dubois et al., 2008), *Bulinus forskalii* (Gow et al., 2001) and *B. aeruginosa* (Gu et al., 2015). The PIC (value of polymorphism) detected by a marker (Nagy et al., 2012), in this study revealed high informative levels of the SSRs as described by Botstein et al. (1980). This information and the high mean values of Ho (observed) and He (expected) indicate a high level of genetic diversity of the *Radix* sp. population in Iraq. However, the value of Ho was lower than He suggesting that an inbreeding situation likely occurred resulting in a deficit of heterozygosity within the *Radix* sp. population. In addition, loci showed significant deviations (P<0.05) from Hardy-Weinberg equilibrium indicating the presence of null alleles at theses loci (Ducarme et al., 2008). No evidence for linkage disequilibrium was detected for these loci suggesting that there is no linkage for all pairs

Table 5. Nei's (1972) coefficients of genetic distance between *Radix* sp. and genus *Melanopsis*.

Species	*Radix* sp.	*M. costata*	*M. nodosa*	*M. buccinoidea*
Radix sp.	1.000			
M. costata	0.434	1.000		
M. nodosa	0.491	0.073	1.000	
M. buccinoidea	0.482	0.518	0.223	1.000
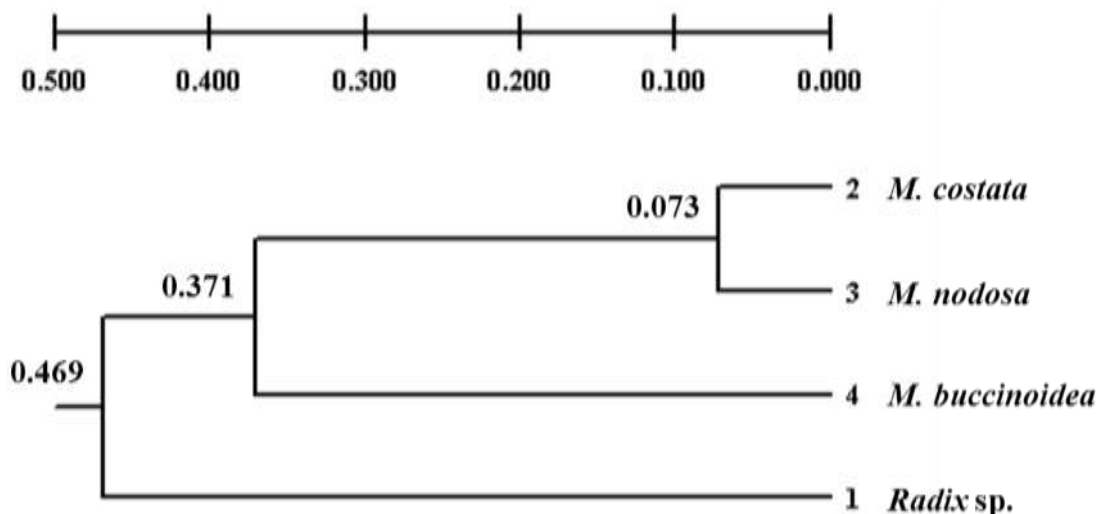
Figure 4. The UPGMA dendrogram based on Nei's genetic distance showing the genetic relationship between *Radix* sp. and *Melanopsis* species.

of loci examined (Salinger and Pfenninger, 2009).

Previous studies have reported that SSR markers are able to cross-amplify loci in related species/genera in plants and animals (BarbarÁ et al., 2007). Successful amplification using SSR between families was about 33% which was lower than comparing between species and between genera (BarbarÁ et al., 2007). No reports of cross amplification across families in freshwater and land snails are available. *Radix* sp. belongs to family Lymnaeidae, while *Melanopsis* spp. belongs to family Melanopsidae. Polymorphic SSR primers from *Radix* sp. in this study were assessed for their transferability to the genus *Melanopsis*; *M. costata* (40%), *M. nodosa* (60%) and *M. buccinoidea* (40%), and showed to be fully transferrable across families. The results showed that one *M. buccinoidea* (No. 37) was grouped with *Radix* sp., while samples No. 9 of *Radix* sp. was included with *Melanopsis* spp. This may be due to missing data of some loci that could affect the clustering; however, most of the samples were clustered together in the same genus. This is the first report of cross-amplification between families of freshwater snails and indicates that the newly developed SSRs are able to be applied as an assessment of the genetic diversity in *Melanopsis* sp. In addition, the markers might be applicable to other groups of freshwater snails.

The SSR markers developed in this study showed a high level of polymorphism in *Radix* sp. collected from the middle and south of Iraq, as well as with freshwater snails of genus *Melanopsis*. This study carried out for the first time the genetic diversity and relationship among *Radix* sp. and high genetic variation within this species in individuals from the middle and south of Iraq was observed. The study demonstrated that SSR markers are useful for further study of the population genetics and evolutionary relationship of *Radix* sp. and other related species.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Al-Waaly ABM, Mohammad KM, Al-Miali HM (2014). Freshwater snails diversity in the middle and south regions of Iraq. Advances in Bioresearch 5(3):166-171.

BarbarÁ T, Palma-Silva C, Paggi GM, Bered F, Fay MF, Lexer C (2007). Cross-species transfer of nuclear microsatellite markers: potential and limitations. Molecular Ecology 16(18):3759-3767.

Belfiore NM, May B (2000). Variable microsatellite loci in red swamp crayfish, *Procambarus clarkii*, and their characterization in other crayfish taxa. Molecular Ecology 9(12):2230-2234.

Benbouza H, Jacquemin JM, Baudoin JP, Mergeai G (2006). Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. Biotechnology, Agronomy, Society and Environment 10:77-81.

Botstein D, White RL, Skolnick M, Davis RW (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. The American Journal of Human Genetics 32(3):314-331.

Carlon DB, LippÉ C (2007). Eleven new microsatellite markers for the tropical sea urchin *Tripneustes gratilla* and cross-amplification in *Tripneustes ventricosa*. Molecular Ecology Notes 7(6):1002-1004.

Caron Y, Lasri S, Losson B (2007). *Fasciola hepatica*: an assessment on the vectorial capacity of *Radix labiata* and *R. balthica* commonly found in Belgium. Veterinary Parasitology 149(1):95-103.

Chbel F, Broderick D, Idaghdour Y, Korrida A, McCormick P (2002). Characterization of 22 microsatellites loci from the endangered Houbara bustard (*Chlamydotis undulata undulata*). Molecular Ecology Notes 2(4):484-487.

Daly-Engel TS, Smith RL, Finn DS, Knoderbane ME, Phillipsen IC, Lytle DA (2012). 17 novel polymorphic microsatellite markers for the giant water bug, *Abedus herberti* (Belostomatidae). Conservation Genetics Resources 4(4):979-981.

Dawson DA, Rossiter SJ, Jones G, Faulkes CG (2004). Microsatellite loci for the greater horseshoe bat, *Rhinolophus ferrumequinum* (Rhinolophidae, Chiroptera) and their cross-utility in 17 other bat species. Molecular Ecology Notes 4(1):96-100.

de Boer MG, Stift M, Michel E (2004). Two new highly polymorphic microsatellite loci and inadvertent minisatellite loci for *Lymnaea auricularia*. Journal of Molluscan Studies 70(1):95-96.

Dubois MP, Nicot A, Jarne P, David P (2008). Characterization of 15 polymorphic microsatellite markers in the freshwater snail *Aplexa marmorata* (Mollusca, Gastropoda). Molecular Ecology Resources 8(5):1062-1064.

Ducarme V, Risterucci AM, Wesselingh RA (2008). Development of microsatellite markers in *Rhinanthus angustifolius* and cross-species amplification. Molecular Ecology Resources 8(2):384-386.

Dung BT, Doanh PN, The DT, Loan HT, Losson B, Caron Y (2013). Morphological and molecular characterization of lymnaeid snails and their potential role in transmission of *Fasciola* spp. in Vietnam. The Korean Journal of Parasitology 51(6):657-662.

Dupuy V, Nicot A, Jarne P, David P (2009). Development of 10 microsatellite loci in the pulmonate snail *Biomphalaria kuhniana* (Mollusca, Gastropoda). Molecular Ecology Resources 9(1):255-257.

Ferté H, Depaquit J, Carré S, Villena I, Léger N (2005). Presence of *Trichobilharzia szidati* in *Lymnaea stagnalis* and *T. franki* in *Radix auricularia* in northeastern France: molecular evidence. Parasitology Research 95(2):150-154.

Gaur A, Singh A, Arunabala V, Umapathy G, Shailaja K, Singh L (2003). Development and characterization of 10 novel microsatellite markers from Chital deer (*Cervus axis*) and their cross–amplification in other related species. Molecular Ecology Notes 3(4):607-609.

Glöer P, Pešić V (2012). The freshwater snails (Gastropoda) of Iran, with descriptions of two new genera and eight new species. ZooKeys219:11-61.

Gow JL, Noble LR, Rollinson D, Jones CS (2001). Polymorphic microsatellites in the African freshwater snail, *Bulinus forskalii* (Gastropoda, Pulmonata). Molecular Ecology Notes 1(4):237-240.

Gu Q, Xiong B, Zhu Y, Wang Q, Shi P (2012a). Isolation and characterization of polymorphic microsatellites loci in the freshwater snail *Bellamya aeruginosa* (Mollusca, Gastropoda). Conservation Genetics Resources 4(2):387-390.

Gu QH, Cheng QQ, Li XJ, Zhou CJ (2015). Novel polymorphic microsatellite markers for *Bellamya* and their application in population genetics of three species. Genetics and Molecular Research 14(4):15201-15212.

Gu QH, Xiong BX, Zhu YT, Wang Q, Shi PS (2012b). Development of 18 microsatellite loci for the freshwater snail *Bellamya aeruginosa* (Mollusca, Gastropoda). Genetics and Molecular Research 11(2):1449-1453.

Gupta PK, Varshney RK (2000). The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. Euphytica 113(3):163-185.

Huang X, Madan A (1999). CAP3: a DNA sequence assembly program. Genome Research 9(9):868-877.

Hubendick B (1951). Recent Lymnaeidae: their variation, morphology, taxonomy, nomenclature, and distribution Almqvist and Wiksell, Kungliga Svenska Vetenskapsakademiens handlingar. Fjarde Serien 3(1):139-144.

Ibarra AM, Petersen JL, Famula TR, May B (2006). Characterization of 35 microsatellite loci in the Pacific lion-paw scallop (*Nodipecten subnodosus*) and their cross-species amplification in four other scallops of the Pectinidae family. Molecular Ecology Notes 6(1):153-156.

Iyengar A, Piyapattanakorn S, Heipel DA, Stone DM, Howell BR, Child AR, MacLean N (2000). A suite of highly polymorphic microsatellite markers in turbot (*Scophthalmus maximus* L.) with potential for use across several flatfish species. Molecular Ecology 9(3):368-371.

Jackiewicz M (1998). European species of the family Lymnaeidae (Gastropoda: Pulmonata: Basommatophora). Genus 9(1):1-93.

Jackiewicz M (2000). Structure of lymnaeid shell columella (Gastropoda: Pulmonata). Malakologische Abhandlungen Staatliches Museum für Tierkunde Dresden 20:37-44.

Knott KE, Puurtinen M, Kaitala V (2003). Primers for nine microsatellite loci in the hermaphroditic snail *Lymnaea stagnalis*. Molecular Ecology Notes 3(3):333-335.

Liu HP, Hershler R (2008). Permanent Genetic Resources: Microsatellite markers for the threatened Bliss Rapids snail (*Taylorconcha serpenticola*) and cross-amplification in its congener, *T. insperata*. Molecular Ecology Resources 8(2):418-420.

Liu K, Muse SV (2005). PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics 21(9):2128-2129.

Martins WS, Lucas DC, Neves KF, Bertioli DJ (2009). WebSat--a web software for microsatellite marker development. Bioinformation 3(6):282-283.

McMullin ER, Wood J, Hamm S (2004). Twelve microsatellites for two deep sea polychaete tubeworm species, *Lamellibrachia luymesi* and *Seepiophila jonesi*, from the Gulf of Mexico. Molecular Ecology Notes 4(1):1-4.

Miller MP (1997). Tools for population genetic analysis (TFPGA) 1.3. A windows program for the analysis of allozyme and molecular population genetic data. http://www. marksgeneticsoftware. net/.

Mohammad MK (2014). Ecology of the freshwater snail *Melanopsis buccinoidea* (Olivier, 1801) in Ain Al-Tamur, Kerbala Province. International Journal of Current Microbiology and Applied Sciences 3:390-394.

Monsutti A, Perrin N (1999). Dinucleotide microsatellite loci reveal a high selfing rate in the freshwater snail *Physa acuta*. Molecular Ecology 8(6):1076-1078.

Nagy S, Poczai P, Cernak I, Gorji AM, Hegedus G, Taller J (2012). PICcalc: an online program to calculate polymorphic information content for molecular genetic studies. Biochemical Genetics 50(9-10):670-672.

Naser MD (2006). Some notes on Melanopsidae (Gastropoda) of Mesopotamia. Iraqi Journal of Aquaculture 3(2:85-90.

Naser MD, Yasser AG, Al-Khafaji KK, Aziz NM, Gmais SA (2008). The

genus *Lymnaea* (Lamarck, 1799) from southern Mesopotamia: Are the morphological and anatomical studies enough to solve its complexity? Marina Mesopotamica 23:349-362.

Nei M (1972). Genetic distance between populations. The American Naturalist 106:283-292.

Nicot A, Jarne P, David P (2009). Development of polymorphic microsatellite loci in the hermaphroditic freshwater snails *Drepanotrema surinamense* and *Drepanotrema depressissimum*. Molecular Ecology Resources 9(3):897-902.

Nugraha M, Pouyaud L, Carman O, Kadarusman, Widyastuti U, Avarre JC (2014). Development of twelve novel polymorphic microsatellite DNA markers for the Boeseman's rainbowfish (*Melanotaenia boesemani*) and tests for their cross-utility in 21 rainbowfish species from West Papua (Indonesia). European Journal of Wildlife Research 60(6):941-946.

Parine NR, Kumar D, Pathan AAK, Elrobh MS, Khan W, Alanazi M (2013). Characterization and cross-species amplification of microsatellite markers in African Silverbill (*Lonchura cantans*). Genetics and Molecular Research 12(4):5634-5639.

Pfenninger M, Cordellier M, Streit B (2006). Comparing the efficacy of morphologic and DNA-based taxonomy in the freshwater gastropod genus *Radix* (Basommatophora, Pulmonata). BMC Evolutionary Biology 6(1):100.

Powell W, Machray GC, Provan J (1996). Polymorphism revealed by simple sequence repeats. Trends in Plant Science 1(7):215-222.

Raymond M, Rousset F (1995). GENEPOP (Version 1.2): Population genetics software for exact tests and ecumenicism. Journal of Heredity 86(3):248-249.

Salinger M, Pfenninger M (2009). Highly polymorphic microsatellite markers for *Radix balthica* (Linnaeus 1758). Molecular Ecology Resources 9(4):1152-1155.

Seddon MB, Van Damme D, von Proschwitz T, Madhyastha A (2014). *Radix auricularia*. The IUCN red list of threatened species. eT155568A42429613. http://dx.doi.org/10.2305/IUCN.UK.2014-1.RLTS.T155568A42429613.en

Sraphet S, Boonchanawiwat A, Thanyasiriwat T, Boonseng O, Tabata S, Sasamoto S, Shirasawa K, Isobe S, Lightfoot DA, Tangphatsornruang S, Triwitayakorn K (2011). SSR and EST-SSR-based genetic linkage map of cassava (*Manihot esculenta* Crantz). Theoretical and Applied Genetics 122(6):1161-1170.

Sraphet S, Saengsri A, Smith DR, Triwitayakorn K (2015). Genetic analysis of *Cheirostylis* species based on microsatellite markers. Plant Genetic Resources 13(3):286-289.

Stoeckle BC, Müller D, Müller J, Kuehn R (2014). Identification of 10 polymorphic microsatellite loci in the high montane gastropod *Semilimax kotulae* using high-throughput sequence data. Molluscan Research 34(1):20-24.

Trouve S, Degen L, Meunier C, Tirard C, Hurtrez-Bousses S, Durand P, Guegan JF, Goudet J, Renaud F (2000). Microsatellites in the hermaphroditic snail, *Lymnaea truncatula*, intermediate host of the liver fluke, *Fasciola hepatica*. Molecular Ecology 9(10):1662-1664.

Vinarski MV (2013). One, two, or several? How many lymnaeid genera are there? Ruthenica 23(1):41-58.

VyskoČllovÁ M, OndraČKovÁ M, ŠlmkovÁ A, Martin J-F (2007). Isolation and characterization of microsatellites in *Neogobius kessleri* (Perciformes, Gobiidae) and cross-species amplification within the family Gobiidae. Molecular Ecology Notes 7(4):701-704.

Wilmer JW, Hughes JM, Ma J, Wilcox C (2005). Characterization of microsatellite loci in the endemic mound spring snail *Fonscochlea accepta* and cross species amplification in four other hydrobiid snails. Molecular Ecology Notes 5(2):205-207.

Zane L, Bargelloni L, Patarnello T (2002). Strategies for microsatellite isolation: a review. Molecular Ecology 11(1):1-16.

African Journal of Biotechnology

Full Length Research Paper

Antioxidant activity of essential oil of three cultivars of *Amomum subulatum* and standardization of high performance thin layer chromatography (HPTLC) method for the estimation of 1,8-cineole

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Essential oils of the fruits of three cultivars of *Amomum subulatum* (Family-Zingiberaceae), such as varlangy, seremna and sawney were isolated. Antioxidant potential was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging and $FeCl_3$-reducing models and the presence of 1,8-Cineole was quantified using the developed high performance thin layer chromatography (HPTLC) method. In the DPPH scavenging model, the IC_{50} value of the essential oil of the seremna, varlangy and sawney were found to be 172.3, 216.9 and 274.3 μg/ml, respectively, while in the ABTS scavenging model, the IC_{50} value of the seremna, varlangy and sawney were found to be 27.96, 31.34 and 32.49 μg/ml respectively. The antioxidant power of the $FeCl_3$ reducing model, the absorbance value of the essential oil of the seremna was found comparatively higher than the essential oils of varlangy and sawney. The content of 1,8-cineole was determined by the developed HPTLC densitometric method, and the value of the percentage (mean ± SD, % w/w) content of the essential oils of the seremna, varlangy and sawney were found to be 69.59 ± 1.45%, 48.78 ± 3.21 and 47.84 ± 1.76 respectively. The highest antioxidant value of seremna cultivar may be due to the presence of high content of 1, 8-cineole. The HPTLC method, therefore confirms that monoterpene 1,8-cineole is the main antioxidant compound present in the fruits of *A. subulatum*.

Key words: *Amomum subulatum*, cultivars*,* essential oil, 1,8-cineole*,* high performance thin layer chromatography (HPTLC), antioxidants.

INTRODUCTION

The fruits of *Amomum subulatum* or greater cardamom is one of the most expensive fruits of the large family, Zingiberaceae of the Kingdom Plantae (Kumar et al., 2013). It is a perennial herb consisting of subterranean rhizomes and leafy aerial shoots which stands erect at 1.7 to 2.6 m depending on the cultivars. Inflorescence of this plant has a condensed spike, commonly found, and has 10-40 fruits in each spike depending on the cultivars. Flowering season starts during March–April at lower altitudes, and in May, at higher altitudes. Harvesting starts during August–September at lower altitudes and October–December at higher altitudes (Gupta and John,

1987). It is also known by other common names such as, English name: black cardamom, hil cardamom, Bengal cardamom, Nepal cardamom; Sanskrit: Aindri, Sthula ela, Brihatupakunchika, Hindi, Punjabi and Gujarati kali ilaichi; Malayalam: karutta elakka; Marathi: Moto-eldori, Moteveldode and Thorveldoda. There are a number of cultivars of *A. subulatum* species grown worldwide, with some of the cultivars such as ramsey, sawney, golsey, varlangy, seremna, etc. popularly cultivated in the Sikkim State and North Bengal, Darjeeling (West Bengal State) of India. Sikkim is one of the leading states in India, which produce huge quantity of the fruits of *A. subulatum* (Dubey and Yadav, 2001). In the area, Sikkim ranked 28th; situated in the north eastern side of India and contributes approximately 53% of the world production of the greater cardamom (Sharma et al., 2000; Agnihotri and Wakode, 2010; Joshi et al., 2013). In India, this perennial herb is commonly planted at an elevation of 700 to 1650 m above sea level and is widely used for the condiments, homemade remedies, and Indian and Chinese systems of medicine (Mukherjee, 1972; Madhusoodhanan and Rao, 2001). In addition to India, the other cultivars of *A. subulatum* is also cultivated in the several other countries such as Nepal, Bhutan, China and Indonesia.

Dried fruits of spices and its essential oils are a good source of an antioxidants. Commercial synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are used to avert lipid peroxidation of commercial food products but these chemicals also which have carcinogenic properties. The essential oil of *A. subulatum* has already been used as a preservative in the juice industry (Kapoor et al., 2009). The essential oil of the fruits of Greater cardamom has already been evaluated for the antioxidant and other activities (Sharma et al., 2017). The other activities such as antiviral, antitussive, antiallergic, anti-inflammatory, bronchodilator, mucolytic, gastroprotective, antitumor, antimicrobial and insecticidal activities of the 1,8-cineole has also been reported (Juergens et al., 2003; Lima et al., 2013). Essential oil obtained from the fruits of *A. subulatum* using hydro-distilled methods, mainly contain monoterpenes 1,8-cineole (Kaskoos et al., 2013). Along with 1,8-cineole, the other monoterpenes such as α-terpineol, DL-limonene, nerolidol, 4-terpineol, δ-terpineol, δ-3-carene, β-myrcene, germacrene D, α-terpinene and longifolenaldehyde have been reported in the hydrodistilled essential oil of the fruit (Vilela et al., 2009; Hussain et al., 2011).

Nowadays, research is concerned with finding the safe and natural antioxidant for the commercial food products to avoid the carcinogenicity of the food products linked with synthetic antioxidants, but in the most of the investigations, the natural antioxidants showed comparatively lower potency than synthetic antioxidants (Ramalho and Jorge, 2006). There is need to investigate the antioxidant potential of the natural sources in the whole extract, because in addition to the principal compounds several other agonist and antagonist compounds present in it, which may together show synergistic potential or other compounds may revert the foremost side effects (Jimenez-Andrade et al., 2003). The oxidative stress is an imbalance between free radical production and antioxidant defences, due to overproduction of free radical through metabolic process and caused several diseases (Uttara et al., 2009; Lobo et al., 2010). Numerous research suggests that consumption of oils, fruits and vegetables or other natural products are important for decreasing the ROS oxidative stress by a variety of mechanisms (Rahal et al., 2014). HPTLC is one of the most advanced form of chromatography, highly applicable for the quantification of active compounds in the extracts, oils and other natural products and it is widely accepted in all over the world (Attimarad et al., 2011; Kathirvel et al., 2012). The main purpose of this study was to select a highly antioxidant varieties of fruits of A. *subulatum* species following *a* new approach, that is, comparing the essential oil of three popular varieties of *A. subulatum fruits,* using DPPH, ABTS and ferric reducing methods along with quantifying the main active constituents 1-8 Cineole using newly developed, reproducible and accurate HPTLC method.

MATERIALS AND METHODS

Three different cultivars of *A. subulatum* fruits (sawney, seremna and varlangy) were obtained from ICRI Tadong, Sikkim, India, in 2016. The reagents such as DPPH, ABTS and BHT (3,5-ditert-4-butylhydroxy toluene were obtained from Sigma, USA. The markers 1,8 cineole (purity > 99%) was procured from the supplier (Sigma Aldrich), Polaris Bioscience, Delhi, India. The pre-coated aluminium-backed TLC plates (silica gel, 0.2 mm thick, 60F$_{254}$, 20 × 20 cm) were purchased from E. Merck, Germany.

Separation of essential oil

The fruits of sawney, seremna and varlangy cultivars of *A. subulatum* were coarsely powdered in the mixer grinder and passed through sieve No. 60. About 100 g of each powder was hydro-distilled for 4 h using Clevenger apparatus (lighter than water). Further, distilled essential oil was collected and dried over anhydrous sodium sulphate; the obtained essential oils were transferred using a glass vial, and stored in the deep freezer for further study.

Antioxidants activity

Free radical scavenging activity (DPPH model)

The DPPH scavenging activity of the essential oil was assessed by using reported method (Asnaashari et al., 2016), with slight modification. In short, essential oils and standard (BHT) were separately diluted in methanol to obtain 1000 µg/ml stock solution. 80 µg/ml of DPPH solution was prepared. Six dilutions (500 to15. 6 µg/ml) of samples and standard were prepared separately. Accurately, 5 ml of DPPH was mixed with 5 ml of samples and

standard solution and the mixtures were kept in the dark at 25°C for 30 min. The absorbance was recorded immediately at 517 nm by UV spectrophotometer. Percentage inhibition of free radical was calculated by the following formula:

$$\% \text{ inhibition} = \frac{B - A}{B} \times 100$$

Where, A was the absorbance of sample and standard solution and B was the means absorbance of blank (without essential oil or standard, BHT). Further, a plot between percentage inhibition and concentration of different dilutions of samples and standards was plotted for the estimation of IC_{50} (50% inhibition).

Free radical scavenging activity (ABTS model)

The ABTS antiradical activity of essential oils and the standard was assessed using reported methods (Yang et al., 2009) that was slightly modified. In short, 1000 µg/ml stock solution was used to prepare the serial dilutions (200 - 12.5 µg/ml) of samples and standard. The ABTS solution was prepared by reacting equal amount of ABTS (7 mM) with potassium persulfate (2.45 mM). The reaction mixture was kept in the dark for at least 4 h at 25°C. Further, it was diluted with 0.1 M sodium phosphate buffer (pH 7.4) to acquire an absorbance of 0.70 ± 0.02 at 734 nm. Accurately, 2.99 ml of ABTS solution was mixed with 10 µl of serial diluted solution of samples and standard and kept in the dark at 25°C for 30 min. An absorbance was recorded immediately at 734 nm by UV spectrophotometer. Percent inhibition and IC_{50} were calculated by equation mentioned in DPPH assay.

Reducing power assay (Ferric chloride model)

The reducing power assay of the essential oils was assessed using reported methods (Olugbami et al., 2015) with slight modification. In short, a stock solution of samples and standard were used for the serial dilutions (200 - 25 µg /ml). About 1 ml of solution from each dilution was mixed with 2.5 ml of phosphate buffer of 0.2 mol/l (pH 6.6) and 2.5 ml of 1% $C_6N_6FeK_3$ solution and were incubated at 50°C for 30 min. About 2.5 ml of 10% trichloroacetic acid (TCA) solution was added into it and was then centrifuged for 10 min at 3000 g. Further, about 2.5 ml of the supernatant was pooled with 2.5 ml of distilled water, shaken with 0.5 ml of 0.1% $FeCl_3$ solution (freshly prepared) and incubated at room temperature for 10 min. The absorbance was recorded at 700 nm against blank (n=3). A graph was plotted between the average of absorbance and concentration for estimation of antioxidant activity.

Chromatographic conditions

The high performance thin layer chromatography (HPTLC) method was developed on a system consisting; TLC applicator Linomat V, automatic development chamber 2 (ADC2), derivatization chamber, TLC plate heater, TLC Scanner equipped with WinCATS software (version 1.4.6) and TLC Reprostar 3 (all from Camag, Muttenz, Switzerland). Separation, derivatization and identification were done with *TLC Silica Gel 60 F254 Glass* plates, 20 × 20 cm and derivatization of plates was developed by automatic TLC sprayer using Vanillin-H_2SO_4 reagents.

Preparation of standard and sample solutions

1000 µg/ml w/v of the standard solution of 1,8-cineole, and the sample solution of the essential oils were prepared separately in the solvent methanol.

Chromatographic conditions

Estimation of 1,8 cineole was performed on a TLC plate without pre-washing. Standard and samples of essential oils were applied to the silica gel plates as 8 mm bands with Linomat V. The plates were developed in automatic development chamber 2 to a distance of 80 mm, relative humidity 50-60%, at 25 ± 5°C, using mobile phase composed of hexane: ethyl acetate (8:2), saturation for 25 min. After development, the plates were air-dried and observed in a visualizer at white light, 254 nm and 366 nm. The reagent Vanillin-H_2SO_4 was used for the derivatization and then the plates were heated at 100°C using a TLC plate heater. The plate was then cooled and scanned in the scanner at UV 665 nm for the quantification.

Calibration curve for standard

The calibration curve for the standard was prepared after application of 1 to 7 µg on a TLC plate. It was then developed and scanned as per the conditions of chromatography. The peak areas were recorded and the calibration curve of standard 1,8-cineole was made by plotting peak area verses concentration.

Method validation

The intended method was validated, referring to the International Conference on Harmonization guidelines (ICH, 2005). The linearity of the current method for the 1,8-cineole was checked (100 to 700 ng/spot) and concentration was plotted against peak area. LOD (Limit of detection) and LOQ (limit of quantification) were determined from the slope of the calibration curve. The equation $3.3 \times SD \times S^{-1}$ and $10 \times SD \times S^{-1}$ were used for the determination LOD and LOQ respectively. The accuracy of the present method was tested by the pre examined samples spiked with 1,8-cineole (0, 50, 100, and 150%); the mixtures were re-examined; and the percentage of recovery and RSD (% Relative standard deviation) were calculated. The precision of standard was determined by study of repeatability and intermediate precision. Repeatability precision was measured in 6 replicates of a standard solution at three different concentration levels 300, 400 and 500 ng/spot on the same day (intra-day precision). Intermediate precision was also measured in 6 replicates of a standard solution at three different concentration levels on different days (inter-day precision). Robustness of the method was determined by estimating the effect on small changes in the polarity of the mobile phase.

Statistical data

The mean ± standard deviation (SD), relative to percentage of scavenging activity by means of non-linear regression, followed by log (inhibitor) vs. response - Variable slope (four parameters) was used to estimate the IC_{50} using software GraphPad Prism, version 7.03 (San Diego, CA).

RESULTS AND DISCUSSION

DPPH free radical scavenging activity

Figure 1 shows the percentage DPPH radical scavenging
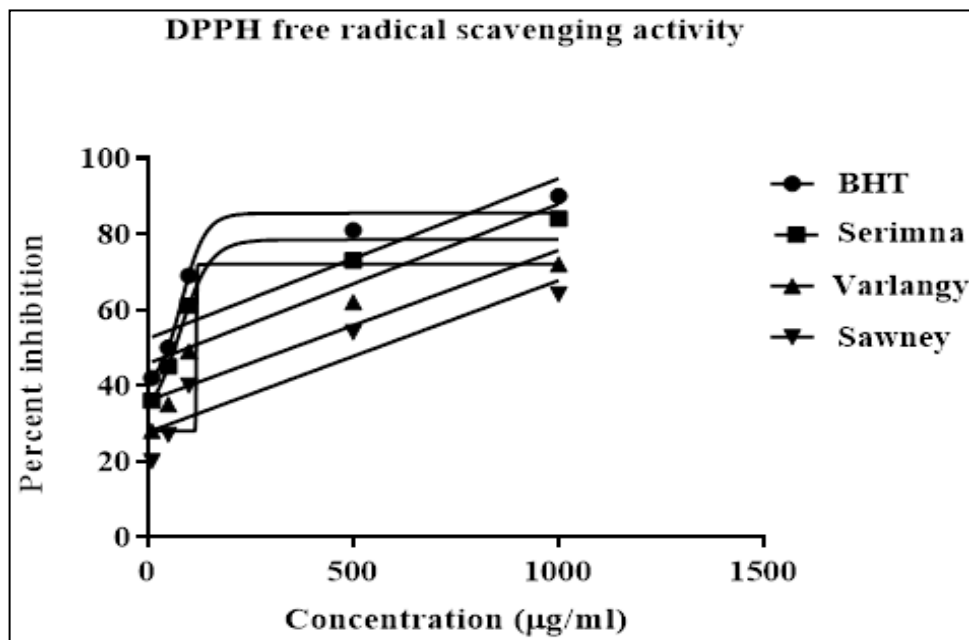
Figure 1. DPPH scavenging activity of essential oils obtained from the fruits of seremna, varlangy and sawney. Values are expressed as mean ± SEM (n=3).

Table 1. Antioxidant activity of essential oils obtained from the fruits of seremna, varlangy and sawney determined using three different methods such as DPPH, ABTS and $FeCl_3$-reducing models.

Essential oils	DPPH method	ABTS method	$FeCl_3$-reducing method
	IC_{50} (µg/ml)		Absorbance
Seremna	172.3	27.96	1.453
Varlangy	216.9	31.34	1.011
Sawney	274.3	32.49	1.001
BHT	84.54	22.77	1.858

activity of the essential oil of three different cultivars of *A. subulatum* fruits and result revealed the different levels of scavenging activities. The percentage scavenging of free radical by Seremna was 84.74% comparatively higher than for varlangy (72.38%) and sawney (64.42%). Table 1 shows the IC_{50} value of essential oils and standard; 84.54, 172.3, 216.9 and 274.3 µg/ml of BHT, seremna, varlangy and sawney respectively. The previous study confirmed that the *in vitro* DPPH free radical scavenging assay is a reliable and stable method for the investigation of antioxidant activity (Amiri, 2012). Pathogenesis of various diseases were caused by free radicals, which are produced during metabolic reaction. Free radicals can oxidize all types of biomolecules, spread in all the body parts and damage cells. The antioxidant compounds that are present in extracts or essential oil of spices and herbs scavenge the free radical and prevent the various diseases. The DPPH antioxidant study of essential oil of fruits of *A. subulatum* has been previously reported by

some of the investigators (Bisht et al., 2011; Dhuley, 1999; Kapoor et al., 2008), but as per our knowledge, this is the first report pertaining to the comparative antioxidant activity of essential of fruits of three cultivars. DPPH and ABTS methods have been widely used for the study of the essential oils (Ballester-Costa et al., 2017; Afoulous et al., 2013). The most powerful scavenging constituent by DPPH was found in the essential oils of seremna cultivar.

ABTS free radical scavenging activity

Figure 2 shows the different levels of ABTS radical scavenging activity in the essential oil of each selected cultivar. The percentage scavenging of essential oil of fruits of seremna was 84.1% comparatively higher than for varlangy (52.6%) and sawney (44.7%). Table 1 shows the IC_{50} value of essential oils and standard such as
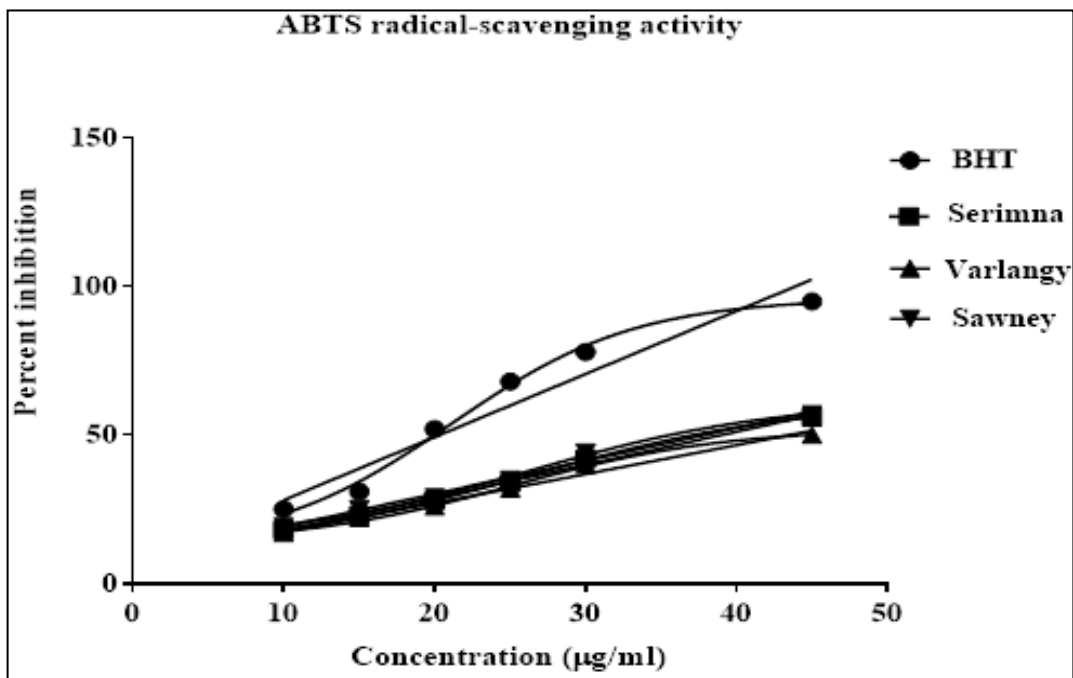
Figure 2. ABTS scavenging activity of essential oils obtained from the fruits of seremna, varlangy and sawney. Values are expressed as mean ± SEM (n=3).
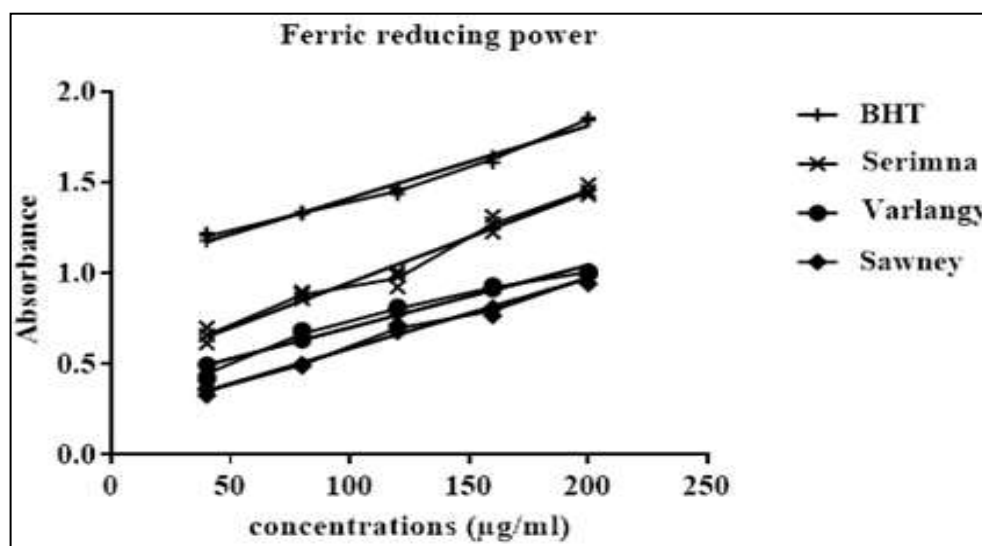
Figure 3. Ferric chloride reducing assay of essential oils obtained from the fruits of seremna, varlangy and sawney. Values are expressed as mean ± SEM (n=3).

27.96, 31.34 and 32.49 µg/ml of seremna, varlangy and sawney respectively. Like DPPH, the ABTS radical scavenging activity also reproduces hydrogen-donating ability. ABTS method is commonly used for the antioxidant profiling of the essential oils; it is a method that is generally superior to DPPH especially for the essential oil activity, due to the complexity and polarity of compounds (Okoh et al., 2016; Kaviarasan et al., 2007).

The most powerful scavenging using the ABTS method was found in the essential oils of seremna cultivar, showing IC_{50}, 27.96 µg/ml.

Ferric chloride reducing assay

Figure 3 shows the reductive capabilities of the essential
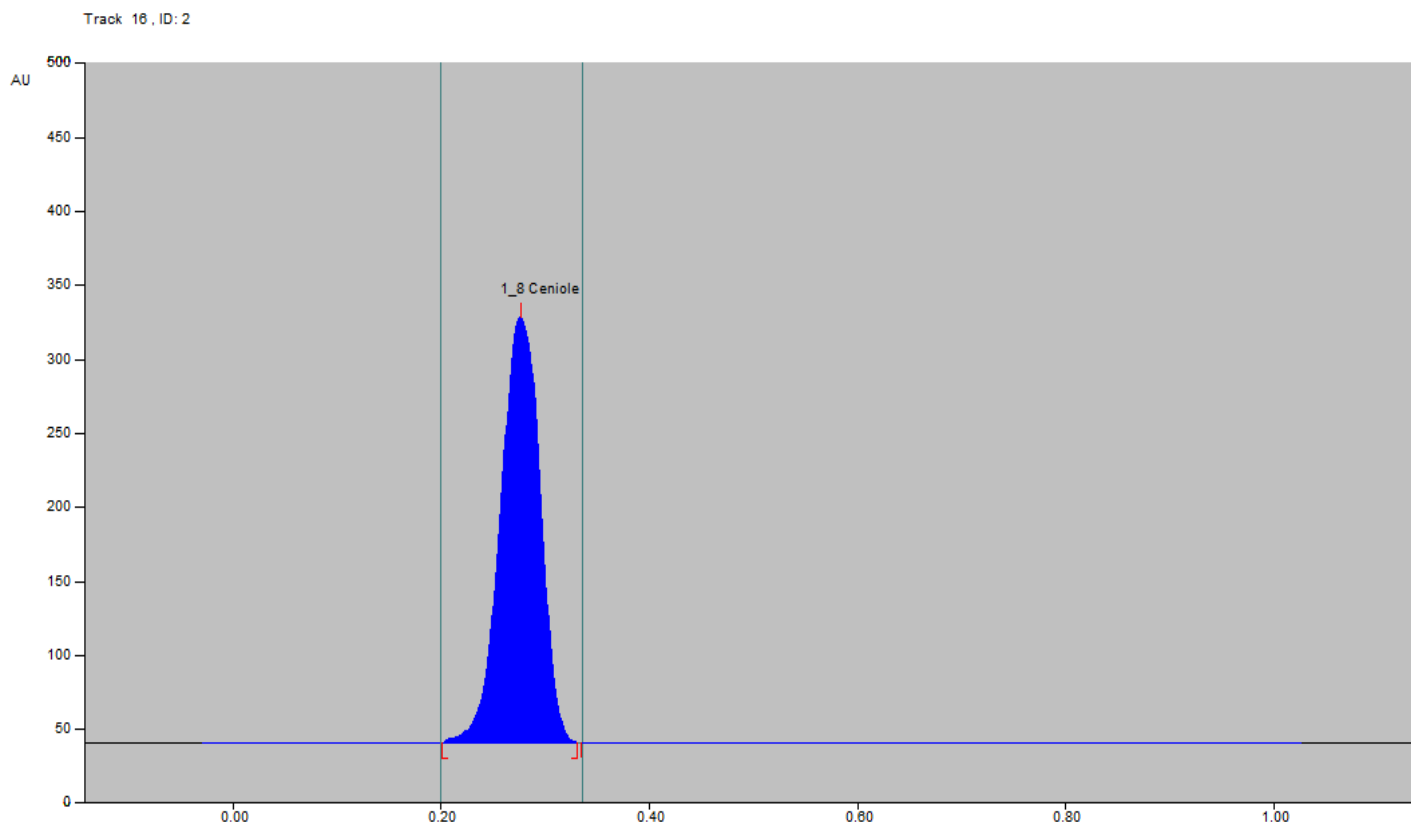
Figure 4. HPTLC densitogram of 1-8 cineole at UV 665 nm.

oils of fruits of cultivars of *A. subulatum* cultivars compared with BHT and the results showed different reducing levels. The reducing power of the essential oil of seremna fruit, 1.453 was found to be higher than the varlangy, 1.011 and sawney, 1.001 at 200 μg/ml, which increased gradually with a rise in the concentration. The method of reducing power assay based on the reaction between antioxidant compounds and potassium ferric cyanide to form potassium ferrous cyanide, which then reacts with ferric chloride to form complex mixture of ferric–ferrous that has an absorption maximum at 700 nm. The reducing power of the essential oils and BHT increases with the increase in the concentration of antioxidant compounds (Bhalodia et al., 2013).

Method validation

Selection of mobile phase

Figure 4 shows the HPTLC densitogram of the developed method for the standard 1,8-cineole. For the optimization of mobile phase, different trials were made using several solvents alone and in different proportions. Solvent system consisting of hexane and ethyl acetate was used in the ratio of 8:2, v/v, where a spot was observed at the

R_f value 0.28±0.12 for the standard.

Specificity

The specificity of the method was determined by analysing samples and standard spectra at the peak apex, peak start and end positions of the peak. When excess standards were added to the essential oil of fruits of cultivars to check the specificity of the method, the spectro-densitogram of standard 1,8-cineole and essential oil at λ 665 nm was observed. A satisfactory peak purity was obtained and it was observed that the other essential oil present in the essential oil did not interfere with the standard peak and it was found that chromatogram was stabled in solution and on the TLC plate at room temperature.

Linearity

Table 2 shows the linearity of the developed method, for which a calibration curve of five dilution was prepared by plotting peak area against concentrations. It was evaluated by applying different concentrations of 100 to 700 ng/spot for standard solution 1,8-cineole. A good

Table 2. Linear regression data for the calibration curve (n=6).

Parameter	Value
Linearity range (ng/spot)	100-700
Regression equation	Y = 12.34x + 214.9
Correlation coefficient	0.9977
Slope ± SE	12.34 ± 0.2664
Intercept ± SE	214.9 ± 119.1
95% Confidence Interval of slope	11.66 to 13.03
95% Confidence Interval of intercept	521.1 to 91.36
F	2147
DFn/DFd	1, 5
P value	< 0.0001
LOD	5.14 ngband^{-1}
LOQ	14.66 ngband^{-1}

Table 3. Accuracy of the proposed method (n=6).

Excess drug added to analyte (%)	Theoretical content (ng)	Concentration found (ng) ± SD	% Recovery	% RSD
0	200	196.17 ± 1.94	98.08	0.99
50	300	294.00 ± 2.45	98.00	0.83
100	400	397.33 ± 1.03	99.33	0.26
150	500	494.00 ± 4.00	98.80	0.81

Table 4. Precision of the proposed method (n=6).

Concentration (ng/spot)	Precision	Average concentration ± SD (n = 6)	Standard error (SE)	% RSD
300		3567.00 ± 30.77	12.56	0.86
400	Repeatability precision (Intraday)	4545.40 ± 19.21	7.84	0.42
500		6055.80 ± 27.58	11.26	0.46
300		3573.00 ± 42.15	17.21	1.18
400	Intermediate precision (Interday)	4537.40 ± 28.81	11.77	1.64
500		6037.80 ± 41.41	16.91	0.69

linear relationship was found with r^2 value of 0.9977, intercepts 214.9 ± 119 and slopes 12.34 ± 0.2664, that endorse the accuracy of the present method.

LOD and LOQ

Table 2 also shows the limit of detection (LOD) and limit of quantification (LOQ) of the developed method for the identification of 1,8-cineole. The LOD 5.14 ng/b and LOQ, 14.66 ng/b respectively, were calculated for standard solution and validate the sensitivity of the present method.

Accuracy

Table 3 shows the accuracy of the present method; for

this the pre-analysed samples were spiked with 50, 100 and 150% of the standard 1,8-cineole and the mixtures were re-analysed using the proposed method. The range of percentage recovery (98 to 99.33%) and percentage relative standard deviation, (0.26 to 0.99 % RSD) for standard 1,8-cineole endorse the accuracy of the present method.

Precision

Table 4 shows the precision data on the intraday and inter-day variation for three different concentration levels. The results of repeatability were stated in terms of the relative standard deviation (% RSD). The low % RSD showed the method is precise for the analysis of 1,8-cineole.

Table 5. Robustness of the proposed HPTLC method.

Concentration (ng/spot)	Original*	Used	Changed	Area ± SD (n = 3)	R_f	% RSD
		7.9:2.1	-0.1, +0.1	4537 ± 28	0.30	0.61
400	8:2	8:2	0.0	4539 ± 26	0.28	0.57
		8.1:1.9	+0.1, -0.1	4545 ± 23	0.27	0.52

Table 6. The contents of 1-8 Cineole in the essential oils obtained from the fruits of Seremna, Varlangy and Sawney determined using developed HPTLC method.

Essential oils of cultivars	Contents (mean ± SD, % w/w)	% RSD
Seremna	69.59 ± 1.45	3.47
Varlangy	48.78 ± 3.21	4.18
Sawney	47.84 ± 1.76	2.53

Robustness

Table 5 shows the robustness of the present method of the introduction of small changes in the composition of the mobile phase and the effects on the results were observed. Low % RSD indicated that the developed HPTLC method is robust.

Quantification of 1,8-cineole in the essential of *A. subulatum* cultivars

Table 6 shows the percentage contents of 1,8-cineole in the essential oils of fruits of seremna, varlangy and sawney and these were analysed using developed HPTLC methods. The contents of 1,8-cineole were 69.59 ± 1.45%, 48.78 ± 3.21% and 47.84 ± 1.76% of the essential oils of fruits of seremna, varlangy and sawney, respectively.

Conclusion

The developed HPTLC method was found to be accurate, specific, precise and easy to use for the quantitative analysis of 1,8-cineole. Quantification of the 1,8-cineole using developed method confirmed that the monoterpenes, 1,8-cineole is a principal ingredient found in the essential oil of fruits of *A. subulatum* cultivars. 1,8-Cineole was found to be higher in seremna cultivar fruit in comparing to varlangy and sawney fruits. So, the seremna fruits obtained essential oil is a good choice of antioxidant cultivar in comparison to the other two cultivars fruit. The comparative antioxidant activity of fruit oil further confirmed the antioxidant nature of monoterpene, 1,8-cineole and the study concluded that the higher the concentration of 1,8-cineole, the greater the value of antioxidant. This method can be adopted for the quality control of essential oils and formulations that contains 1,8-ineole as active compounds as well as for the selection of good antioxidant cultivars that contain 1,8-cineole as an active marker.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

REFERENCES

Afoulous S, Ferhout H, Raoelison EG, Valentin A, Moukarzel B, Couderc F, Bouajila J (2013). Chemical composition and anticancer, antiinflammatory, antioxidant and antimalarial activities of leaves essential oil of *Cedrelopsis grevei*. Food and Chemical Toxicology 56:352-362.

Agnihotri S, Wakode S (2010). Antimicrobial activity of essential oil and various extract og greater cardamom. Indian Journal of Pharmaceutical Sciences 72:657-659.

Amiri H (2012). Essential Oils Composition and Antioxidant Properties of Three Thymus Species. Evidence-Based Complementary and Alternative Medicine 2012.

Asnaashari S, Afshar FH, Ebrahimi A, Moghadam SB, Delazar A (2016). Chemical composition and radical scavenging activity of essential oil and methanolic extract of *Eremostachys azerbaijanica* Rech.f. from Iran. Research in Pharmaceutical Sciences 11:113-119.

Attimarad M, Ahmed KKM, Aldhubaib BE, Harsha S (2011). High-performance thin layer chromatography: A powerful analytical

technique in pharmaceutical drug discovery. Pharmaceutical methods 2:71-75.

Ballester-Costa C, Sendra E, Fernández-López J, Pérez-Álvarez JA, Viuda-Martos M (2017). Assessment of Antioxidant and Antibacterial Properties on Meat Homogenates of Essential Oils Obtained from Four Thymus Species Achieved from Organic Growth. Foods 6:59.

Bisht VK, Negi JS, Bhandari AK, Sundriyal RC (2011). *Amomum subulatum* Roxb. traditional, phytochemical and biological activities-A review. African Journal of Agricultural Research 6:5386-5390.

Dhuley JN (1999). Anti-oxidant effects of cinnamon (*Cinnamomum verum*) bark and greater cardamom (*Amomum subulatum*) seeds in rats fed high fat diet. Indian Journal of Experimental Biology 37:238-242.

Dubey AK, Yadav DS (2001). Comparative performance of different varieties of large cardamom (*Amomom subulatum* Roxb.) under mid altitude of Amnachal Pradesh. Journal of Spices and Aromatic Crops 10:119-122.

Gupta U, John TD (1987). 'Floral biology of large cardamom'. Cardamom 20:8-15.

Hussain AI, Anwar F, Nigam PS, Sarker SD, Moore JE, Rao JR, Majumdar A (2011). Antibacterial activity of some Lamiaceae essential oils using resazurin as an indicator of cell growth. LWT-Food Science and Technology 44:1199-1206.

ICH (2005). Harmonized Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology Q2 (R1)Geneva, Switzerland: International Conference on Harmonization.

Jimenez-Andrade JM, Ortiz MI, Perez-Urizar J, Aguirre-Banuelos P, Granados-Soto V, Castaneda-Hernandez G (2003). Synergistic effects between codeine and diclofenac after local, spinal and systemic administration. Pharmacology Biochemistry and Behavior 76:463-471.

Joshi R, Sharma P, Sharma V, Prasad R, Sud RK, Gulati A (2013). Analysis of the essential oil of large cardamom (*Amomum subulatum* Roxb.) growing in different agro-climatic zones of Himachal Pradesh, India. Journal of the Science of Food and Agriculture 93:1303-1309.

Juergens UR, Dethlefsen U, Steinkamp G, Gillissen A, Repges R, Vetter H (2003). Anti-inflammatory activity of 1,8-cineol (eucalyptol) in bronchial asthma: a double-blind placebo-controlled trial. Respiratory medicine 97:250-256.

Kapoor IPS, Singh B, Singh G (2009). Essential oil and oleoresins of cardamom (*Amomum subulatum* ROXB.) as natural food preservatives for sweet orange (Citrus sinensis) juice. Food Process Engineering 34:1101-1113

Kapoor IPS, Singh B, Singh G, Isidorov V, Szczepaniak L (2008). Chemistry, antifungal and antioxidant activities of cardamom (*Amomum subulatum*) essential oil and oleoresins. International Journal of Essential Oil Therapeutics 2:29-40.

Kaskoos RA, Mir SR, Kapoor R, Ali M (2013). Essential Oil Composition of the Fruits of *Amomum subulatum* Roxb. Journal of essential oil-bearing plants 11(2):184-187.

Kathirvel S, Prasad KR, Babu KM (2012). Development and validation of HPTLC method for the determination of mycophenolate mofetil in bulk and pharmaceutical formulation. Pharmaceutical Methods 3:90-93.

Kaviarasan S, Naik GH, Gangabhagirathi R, Anuradha CV, Priyadarshini KI (2007). In vitro studies on antiradical and antioxidant activities of fenugreek (*Trigonella foenum* graecum) seeds. Food Chemistry 103:31-37.

Kumar KMP, Asish GR, Sabu M, Balachandran I (2013). Significance of gingers (Zingiberaceae) in Indian System of Medicine - Ayurveda: An overview. Ancient science of life 32:253-261.

Lima PR, Melo TSd, Carvalho KMMB, Oliveira IBd, Arruda BR, Castro Brito GAd, Rao VS, Santos FA (2013). 1,8-cineole (eucalyptol) ameliorates cerulein-induced acute pancreatitis via modulation of cytokines, oxidative stress and NF-κB activity in mice. Life Sciences 92:1195-1201.

Lobo V, Patil A, Phatak A, Chandra N (2010). Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy Reviews 4:118-126.

Madhusoodhanan KJ, Rao YS (2001). Cardamom (large), in *Handbook of herbs and spices*, vol I, 1st edn, edited by K V Peter (Woodhead Publishing Limited, England) pp. 134-141.

Mukherjee DK (1972). Large cardamom. World Crops 25:31-33

Okoh SO, Iweriebor BC, Okoh OO, Nwodo UU, Okoh AI (2016). Antibacterial and Antioxidant Properties of the Leaves and Stem Essential Oils of *Jatropha gossypifolia* L. BioMed Research International 2016.

Olugbami JO, Gbadegesin MA, Odunola OA (2015). *In vitro* free radical scavenging and antioxidant properties of ethanol extract of *Terminalia glaucescens*. Pharmacognosy Research 7:49-56.

Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S, Dhama K (2014). Oxidative Stress, Prooxidants, and Antioxidants: The Interplay. BioMed Research International 2014:1-19.

Ramalho VC, Jorge N (2006). Antioxidantes utilizados em óleos, gorduras e alimentos gordurosos. Quimica Nova. 29:755-760.

Sharma E, Sharma R, Singh KK, Sharma G (2000). Large Cardamom Farming in the Sikkim Himalaya. Mountain Research and Development 20:108-111.

Sharma V, Lohia N, Handa V, Baranwal M (2017). *Amomum subulatum* seed extract exhibit antioxidant, cytotoxic and immune suppressive effects. Indian Journal of Biochemistry and Biophysics 54:135-139.

Uttara B, Singh AV, Zamboni P, Mahajan R (2009). Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. Current Neuropharmacology 7:65-74.

Vilela GR, Almeida GS, D'Arce MABR, Moraes MHD, Brito JO, da Silva MFGF, CruzSilva S, Piedade SMS, Calori-Domingues MA, Gloria EM (2009). Activity of essential oil and its major compound, 1,8-cineole, from *Eucalyptus globulus* Labill., against the storage fungi *Aspergillus flavus* Link and Aspergillus parasiticus Speare. Journal of Stored Products Research 45:108-111.

Yang S-A, Jeon S-K, Lee E-J, Im N-K, Jhee K-H, Lee S-P, Lee I-S (2009). Radical Scavenging Activity of the Essential Oil of Silver Fir (*Abies alba*). Journal of Clinical Biochemistry and Nutrition 44:253-259.

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Reduction of organic load from palm oil mill effluent (POME) using selected fungal strains isolated from POME dump sites

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Environmental concerns are becoming important global tasks. Palm oil mill effluent (POME) contains oil and grease and also rich in organic matter in the form of total suspended solids which can increase biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of POME. It is generated in huge quantities during the production of crude palm oil and pollutes land, water and atmosphere if left untreated. The current study mainly focuses on evaluating the efficacy of fungal isolates screened and isolated from POME dump sites in the bioremediation of POME. Five fungal species used in the present study were previously isolated by the author from POME dump sites of Pedavegi palm oil mill industry. Out of these, *Emericella nidulans* NFCCI 3643 was proven to be an excellent biological agent in reducing the organic load of POME. The organism showed 80.28% reduction in COD, 88.23% in BOD and 87.34% in oil/grease content at their optimal environmental and nutritional conditions. The mixed cultures showed better reduction efficiency as compared to individual pure cultures. The natural inhabitants of POME dump sites showed their lipolytic ability and *E. nidulans* was found to be an excellent agent in the bioremediation of POME. Fungal isolates in consortium can function better in bioremediating POME than individual pure cultures.

Key words: Palm oil mill effluent (POME), oil and grease, biochemical oxygen demand (BOD), chemical oxygen demand (COD), organic load, *Emericella nidulans*, bioremediation.

INTRODUCTION

Palm oil mill effluent (POME) is the waste that is being released by the palm oil mills during oil extraction process from palm fruit bunches. In recent years, a lot of attention has been drawn towards environmental hazards that are caused by direct release of industrial effluents without proper treatment. It was estimated that about 1.5 tons of POME were generated for a tone of fresh palm fruit bunches during oil extraction process (Ahmad et al., 2003). POME is a thick brown colored liquid rich in total solids, oil and grease (Poh and Chong, 2008; Mahzad et al., 2009). The chemical oxygen demand (COD) and biological oxygen demand (BOD) of POME is also high

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(Pogaku and Sarbatly, 2013). A high concentration of organic matter, COD (45,000 to 65,000 mg/L) and BOD (18,000 to 48,000 mg/L) of POME was reported by Chin et al. (1996). Soleimaninanadegani and Manshad (2014) reported a COD and BOD in the range of 80,100 to 95,000 and 23,400 to 52,100 mg/L, respectively. A COD and BOD of 40,000 to 50, 000 and 20,000 to 25,000 mg/L were reported by Najafpour et al. (2006). In addition to oil and grease, POME also contains various complex polymers like carbohydrates, lipids, proteins, certain minerals and nitrogenous compounds (Ohimain et al., 2013a). Discharge of untreated POME into aquatic bodies can cause dark coloration of water, eutrophication which further makes the water unsuitable for consumption (Tubonimi et al., 2007; Cheng et al., 2010; Foo and Hameed, 2010). The nature of POME also causes odour pollution (Er et al., 2011). A high COD value of POME also distracts the aquatic life (Maygaonkar et al., 2012), resulting in loss of biodiversity (Singh and Pandey, 2009). As direct discharge of such effluents into the environment without proper treatment might cause considerable environmental problems (Cheng et al., 2010; Awotoye et al., 2011; Jameel and Olanrewaju, 2011; Lam and Lee, 2011), there is need to treat POME from palm oil mills before they are discharged into the environment.

In addition, the high level of organic matter of POME as indicated by its high COD, BOD, grease and oil content, serves as good substrate for growth of a wide variety of microbes (Roux-Van Der Merwe et al., 2005; Md Din et al., 2006). POME being rich in oil content also serves as a habitat for several groups of lipase producing microorganisms as well as hydrocarbon degraders (Rahman et al., 2007; Ohimain et al., 2013b). Use of fungi in the bioremediation of POME has drawn the attention of researchers since last two decades, as most of the previous works involved the use of bacteria in the bioremediation of POME. Several fungi like *Rhizopus, Mucor, Candida rugosa, Geotrichum candidum, Aspergillus*, etc. have been well studied for their ability to produce lipolytic enzymes (Burkert et al., 2004; D'Annibale et al., 2006; Grbavcic et al., 2007; Nwauche and Ogbonna, 2011). Though, POME is a major environmental concern, only limited studies were reported its bioremediation (Oswal et al., 2002; Wu et al., 2010; Soleimaninanadegani and Manshad, 2014). Therefore, screening and isolation of fungi from POME dump sites provides an alternative way to clean up environmental pollutants as these microbes use the organic compounds present in the POME as supplements and thereby degrade these substances into simpler compounds like methane, carbon dioxide and water. The bioconversion of POME by microorganisms also has additional advantages in that, it makes the POME to be useful in the production of a variety of compounds such as antibiotics, biofertilizer, solvent, bio-insecticides, biohydrogen, polyhydroxyalkanoates, organic acids and enzymes (Wu et al., 2009). Hence, the present study was carried out to investigate the ability of the selected indigenous fungi in the bioremediation of POME.

The results obtained in the present study clearly demonstrate that POME dump sites are potential sources of lipase producing microorganisms and can be used to treat POME.

MATERIALS AND METHODS

Collection and preservation of POME

POME was collected from Pedavegi palm oil mill in sterile plastic bottles, sealed and transported in ice box to the laboratory. The sample was stored at -20°C until further use. The physico-chemical characteristics of the POME were studied using the standard methods published by APHA (American Public Health Association, 2005).

Characterization of POME and analytical methods

POME collected from Pedavegi palm oil industry was characterized by determining the physicochemical parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD5), total suspended solids (TSS), oil and grease (O&G) and pH. The treatment efficiency was determined by characterizing the POME before and after the treatment. Reduction in organic load COD was determined spectrophotometrically, BOD5 was used to measure the biodegradability, TSS were determined as dry weight (mg/L), partition-gravimetric method (Kirschman and Pomeroy, 1949) was employed to determine oil and grease content and pH meter was used to measure the pH. The above methods were carried out as per the standard procedures described in the Standard Methods for the Examination of Water and Wastewater (Clesceri et al., 1999; APHA, 1995, 2005).

Reduction efficiency (RE %) of COD was defined as the amount of COD that decreased as compared to the initial COD amount. Reduction efficiency (RE %) of BOD in terms of BOD5 was defined as the amount of BOD that decreased as compared to the initial BOD amount. The experiments were carried out in triplicates.

Fungal isolates

Five fungal species out of 12 isolates that were screened and isolated from the POME dump sites of Peda vegi palm oil mill, West Godavari District, A.P., India (Suseela et al., 2014) were selected for POME inoculation. They include *Emericella nidulans* NFCCI 3643, *Trichoderma reesei*, *Trichoderma harzianum*, *Aspergillus niger* and *Aspergillus fumigatus*. The fungi used for POME inoculation were identified based on morphological characteristics and microscopic observation of fungal spores using lactophenol cotton blue staining. For morphological characterization, the fungal isolates were cultivated on czepakdox agar medium. The shape, size, arrangement and development of conidiophores, phialides and conidiospores were studied using the taxonomic tools of Hoog et al. (2000).

Inoculation of sterile POME

The five fungal isolates that showed highest lipase producing activity were selected in the present study to test their applicability

in the bioremediation of POME. The raw POME sample transferred into 250 mL of Erlenmeyer flask was autoclaved at 121°C for 20 min. The cooled autoclaved sample was inoculated with five percent of spore suspension containing 10^6 cells/mL and incubated at 30°C with shaking at 150 rpm. At an interval of 24 h, samples were collected under aseptic conditions up to 5 days and analyzed for BOD5, COD, oil and grease. Control flasks were not inoculated. The efficiency for organic load reduction and the percentage reduction was measured by using the following equation (Piro et al., 2011):

$$\text{Reduction \%} = 100 - \frac{C_{raw} \text{ POME} - C_f}{C_{raw} \text{ POME}} \times 100$$

Where C_{raw} POME is the concentration of COD, BOD5, oil and grease of raw POME and C_f is the concentration of the above said parameters after treatment. All the experiments were performed in triplicates.

Similar experiments were also carried out using mixed cultures of organisms (MC1 (*E. nidulans* + *A. niger* + *A. fumigatus*) and MC2 (*T. harzianum* + *T. reesei*)) to test whether the organisms in group can perform better as compared to individual pure cultures. For preparing mixed cultures, spores of all fungi were mixed in equal ratio and inoculated into sterile POME.

RESULTS AND DISCUSSION

Characteristics of POME

POME collected from the Pedavegi palm oil mill was a thick dark brown colored viscous oily liquid with abhorrent odour. The raw POME contains a BOD of 39,476 mg/L, COD at a concentration of 79,980 mg/L, TSS 15,238 mg/L, oil and grease 209 mg/L and pH 4.28. Similar findings were reported by Najafpour et al. (2006), Vijayaraghavan et al. (2007), AbdulKarim et al. (2011), Lam and Lee (2011) and Bala et al. (2015).

Fungal isolates from POME dump sites

A total of 12 different fungal members were screened and isolated from POME dump sites (Suseela et al., 2014). Out of them, 5 fungal isolates that showed good lipase producing activity were selected for further studies.

Oil and grease removal (%) from POME using selected fungal isolates

The use of fungi and yeast such as *Trichoderma viride*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica* for the treatment of POME has not been extended to the removal of oil and grease (Jameel and Olanrewaju, 2011) despite their high potential in removing COD from POME. This may be due to the fact that these microorganisms are not indigenous to POME. In the present study, the ability of 5 fungal members isolated from POME dump

sites were investigated for the removal of oil and grease from POME.

The removal (%) of oil and grease from POME is shown in Figure 1. From the results, it is evident that reduction efficiency of *Emericella nidulans* was highest with 87.34% followed by *A. niger*, *T. harzianum*, *A. fumigatus* and *T. reesei* with 71.23, 68.21, 61.17 and 59.09%, respectively. There was only 16.85% reduction efficiency with the control indicating the potentiality of our POME isolates in the removal of oil and grease. The results obtained are in agreement with the reports of Oswal et al. (2002) in his work on treatment of POME with *Y. lipolytica* NCIM 3589. A 93.3% reduction in oil and grease was reported by Lan et al. (2009) using *Y. lipolytica* W29.

Reduction efficiency (RE %) of COD using selected fungal isolates (Individual pure cultures)

Figure 2 shows the reduction efficiency of COD for selected fungal isolates. From the results, it is evident that reduction efficiency of *E. nidulans* was highest with 80.28% followed by *A. niger*, *T. harzianum*, *A. fumigatus* and *T. reesei* with 71.08, 64.83, 61.86 and 59.26%, respectively.

There was only 13.88% reduction efficiency with control indicating that the POME isolates are effective in COD reduction. Similar findings were reported regarding COD reduction by El-Bestawy et al. (2005), Takeno et al. (2005), Lan et al. (2009) AbdulKarim et al. (2011), Abass et al. (2012), Mohammed et al. (2014), Soleimaninanadegani and Manshad (2014) and Bala et al. (2015) in their studies using different microorganisms. The present study is significant in understanding the role of fungi in the bioremediation of oil contaminated effluents such as POME.

Reduction efficiency (RE %) of COD using mixed cultures (combination of fungal isolates)

The reduction efficiency of COD by mixed cultures is shown in Figure 3. From the results, it is clearly evident that there is enhanced organic load reduction with mixed cultures as compared to pure cultures and is as follows: MC1 (*E. nidulans* + *A. niger* + *A. fumigatus*) (91.43%) > MC2 (*T. harzianum* + *T. reesei*) (73.14%) > control (17.23%).

This study provides an understanding on the role of mixed cultures in the treatment of waste waters such as those from oil processing industries. The results reported are in good agreement with the results of previous workers who also used mixed cultures for the effluent treatment (Chigusa et al., 1996; Wakelin and Forster, 1997; AbdulKarim et al., 2011).
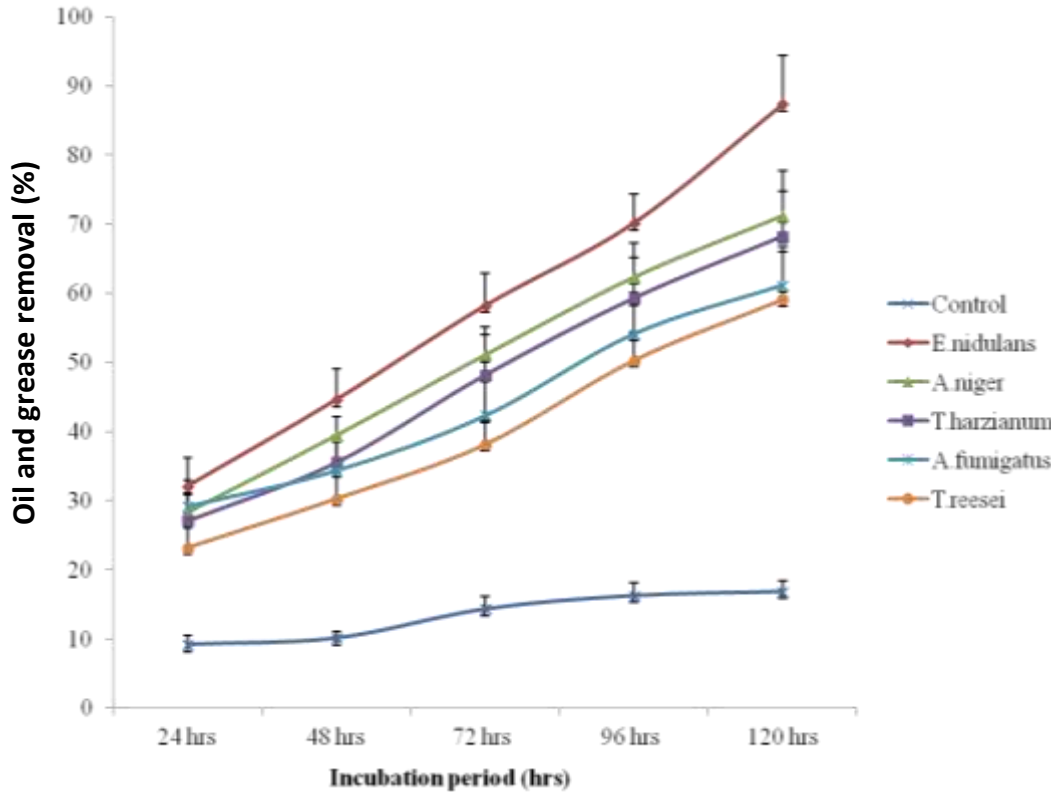
Enhanced organic load reduction with mixed cultures
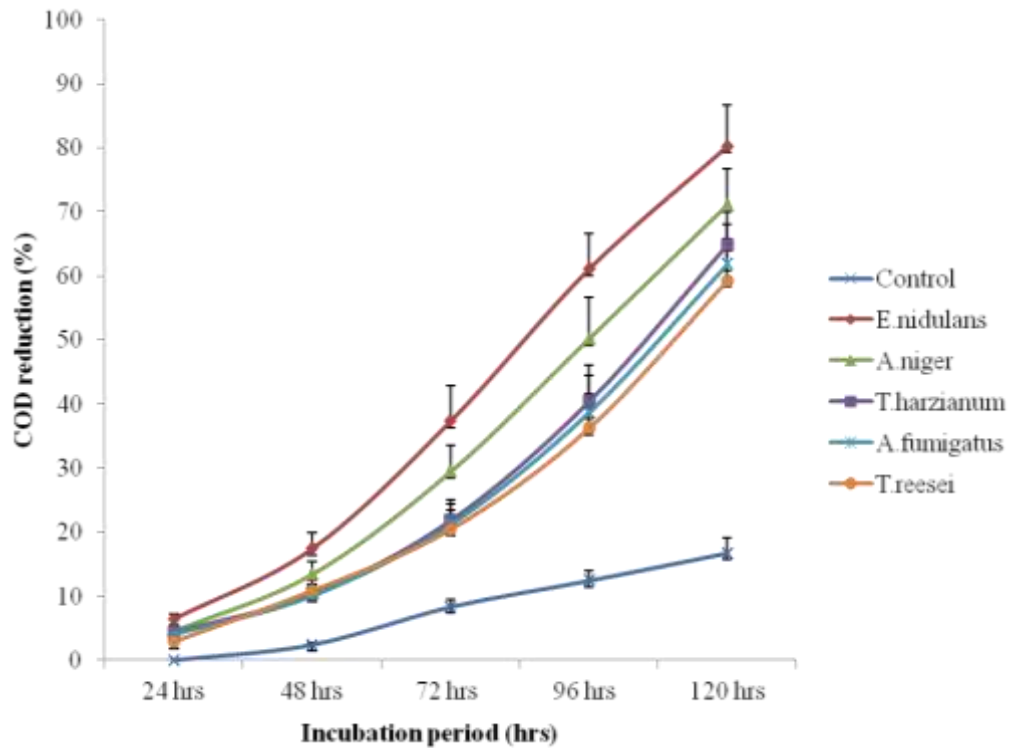
Figure 1. Oil and grease removal (%) in POME.

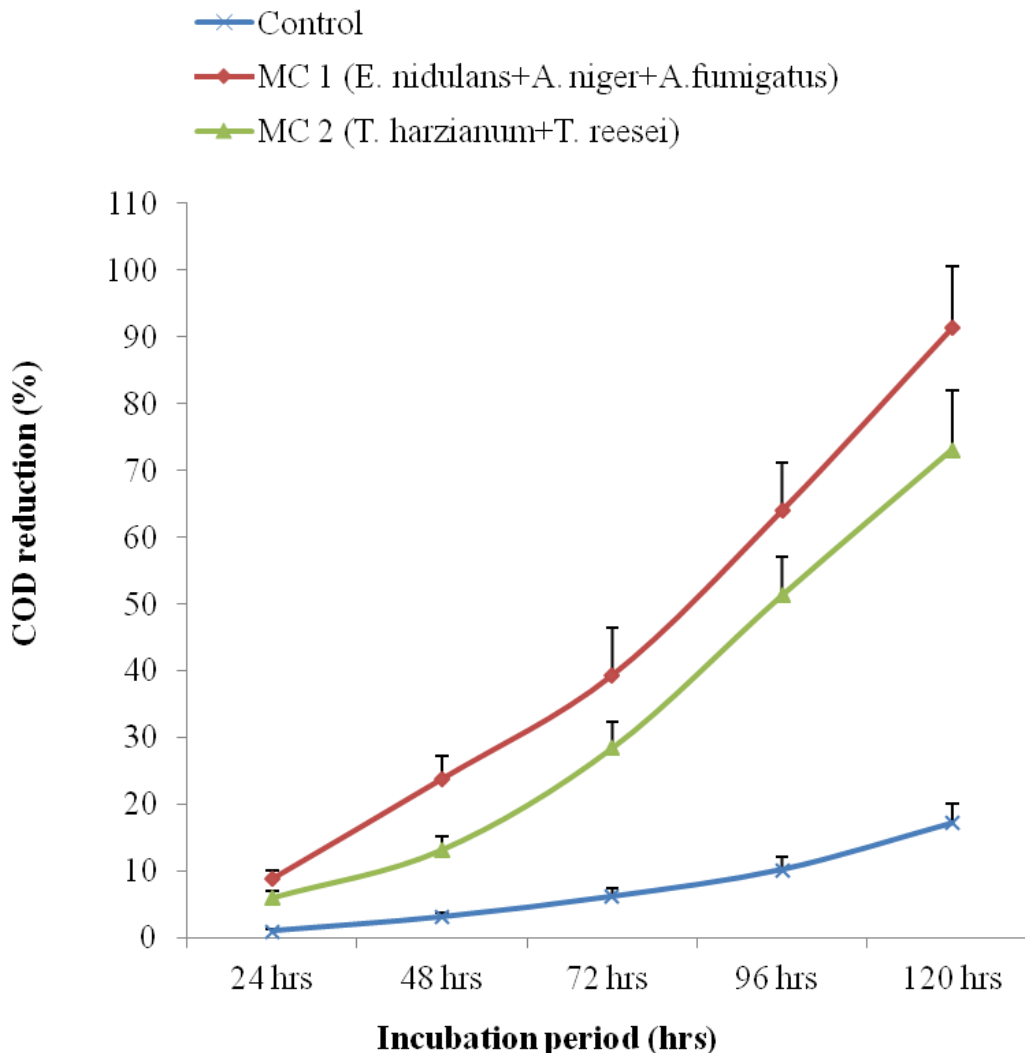**Figure 2.** COD reduction (%) in POME sample (pure cultures).

Figure 3. COD reduction (%) in POME sample (mixed cultures).

was also reported by various workers (El-masry et al., 2004; El-Bestawy et al., 2005). The microorganisms in the mixed cultures utilizes the organic substances of POME as nutrients and hence results in organic load reduction (Jameel and Olanrewaju, 2011; Jameel et al., 2011).

Reduction efficiency (RE %) of BOD using selected fungal isolates (individual pure cultures)

Figure 4 represents the reduction efficiency of BOD for selected fungal isolates. From the results, it is evident that reduction efficiency of *E. nidulans* was highest with 88.23% followed by *A. niger*, *T. reesei*, *A. fumigatus* and *T. harzianum* with 77.64, 68.17, 63.26 and 52.63%, respectively. Similar findings were reported by El-Masry et al. (2004) and El-Bestawy et al. (2005).

Reduction efficiency (RE %) of BOD using mixed cultures (combination of fungal isolates)

The reduction efficiency of BOD by mixed cultures is shown in Figure 5. From the results, it is clearly evident that there is enhanced organic load reduction with mixed cultures as compared to pure cultures and it is as follows: MC1 (*E. nidulans + A. niger + A. fumigatus*) (94.34%) > MC2 (*T. harzianum + T. reesei*) (78.21%) > control (16.54%).

The findings are in agreement with Qingwei et al. (1998) and Bala et al. (2015). The results are mainly attributed to the synergistic effect of different fungal members in the mixed culture (Chigusa et al., 1996; Benka-coker and Ekundayo, 1997; Odegaar et al., 1998). The present treatment process also has advantage in that no additional physical or chemical treatment was required. Similar findings were reported by El-Bestawy et
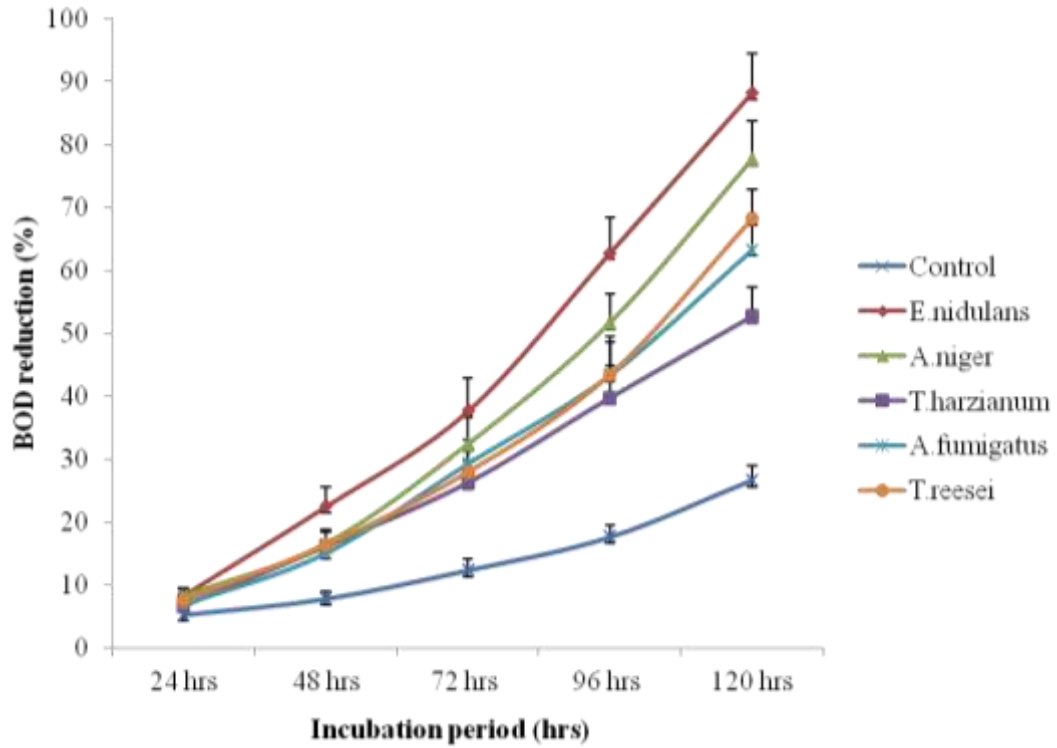
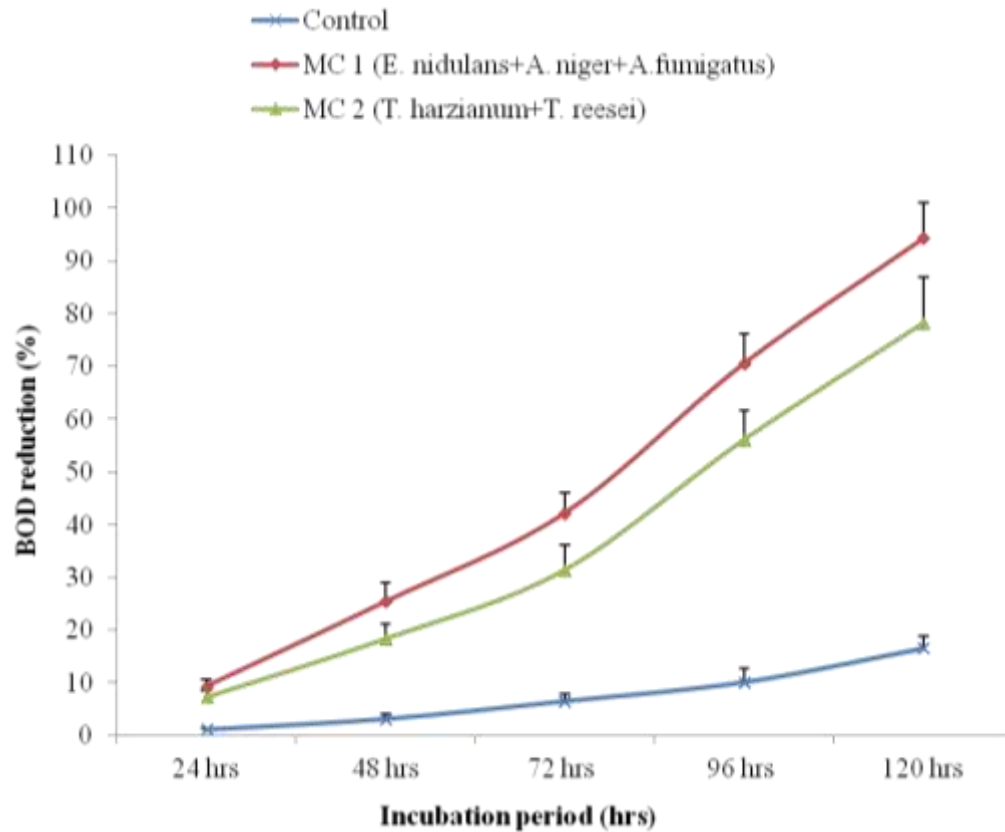**Figure 4.** BOD reduction (%) in POME sample.

Figure 5. BOD reduction (%) in POME sample (mixed cultures).

al. (2005) in his work on treatment of contaminated industrial effluents by mixed cultures of bacteria.

Conclusion

The application of isolated fungi in the biodegradation of POME was investigated in the present study. The 5 fungal members isolated from POME dump sites were found to be effective in reducing COD, BOD, oil and grease of POME. This study is certainly useful in understanding the role of fungal members in either pure cultures or in the form of mixed cultures and in biological treatment of effluents from oil processing industries. The mixed culture (MC1) (*E. nidulans* + *A. niger* + *A. fumigatus*) is found to be most effective in the treatment of POME with a reduction efficiency of COD (91.43%) and BOD (94.34%). As high BOD and COD concentrations of POME make it unsuitable for discharge into the environment, recycling of POME by biological treatment will certainly gain importance based on its safe discharge and reuse.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abass AO, Jameel TA, Muyibi AS, Abdul Karim IM, Alam MdZ (2012). Investigation of the viability of selected microorganisms on the biodegradation of palm oil mill effluents (POME). International Journal of Environmental Science and Technology 3(3):182-186.

AbdulKarim MI, Daud NA, Alam MDZ (2011). Treatment of palm oil mill effluent using microorganisms. In: Alam MDZ, Jameel AT, Amid A (eds) Current research and development in biotechnology engineering at International Islamic University Malaysia (IIUM), 3rd edn. IIUM Press, Kuala Lumpur pp. 269-275.

Ahmad AL, Ismail S, Bhatia (2003). Water recycling from palm oil mill effluent (POME) using membrane technology. Desalination 157(1-3):87-95.

American Public Health Association (APHA) (1995). In: Standard methods for the examination of water and wastewater. American water works association and water pollution control federation. APHA, Washington DC.

American Public Health Association (APHA) (2005). Standard methods for the examination of water and wastewater in 21st edn. American Public Health Association (APHA), Washington.

Awotoye OO, Dada AC, Arawomo GAO (2011). Impact of Palm Oil Processing Effluent Discharging on the Quality of Receiving Soil and Rivers in South Western Nigeria. Journal of Applied Sciences Research 7(2):111-118.

Bala JD, Lalung J, Ismail N (2015). Studies on the reduction of organic load from palm oil mill effluent (POME) by bacterial strains. International Journal of Recycling of Organic Waste in Agriculture 4:1-10.

Benka-coker MO, Ekundayo JA (1997). Applicability of evaluating the ability of microbes isolated from an oil spill site to degrade oil. Environmental Monitoring and Assessment 45:259-272.

Burkert JFM, Maugeri F, Rodrigues MI (2004). Optimization of extracellular lipase production by Geotrichum sp. using factorial design. Bioresource Technology 91:77-84.

Cheng J, Zhu X, Ni J, Borthwick A (2010). Palm oil mill effluent treatment using a two-stage microbial fuel cells system integrated with immobilized biological aerated filters. Bioresource Technology 101(8):2729-2734.

Chigusa S, Hasegawa T, Yamamoto N, Watanabe Y (1996). Treatment of waste-Chappe water from oil manufacturing plant by yeasts. Water Science and Technology 34(11):51-58.

Chin KK, Lee SW, Mohammad HH (1996). A study of palm oil mill effluent treatment using a pond system. Water Science and Technology 34(11):119-123.

Clesceri LS, Greenberg CG, Eaton AD (1999). Standard Method for the Examination of Water and Wastewater. 20th edn. USA: American Public Health Association (APHA).

D'Annibale A, Sermanni GG, Federici F, Petruccioli M (2006). Olive-oil wastewaters: A promising substrate for microbial lipase production. Bioresource Technology 97:1828-1833.

El-Bestawy E, El-Masry MH, El-Adl NE (2005). The potentiality of free Gram-negative bacteria for removing oil and grease from contaminated industrial effluents. World Journal of Microbiology and Biotechnology 21(6-7):815-822.

El-Masry MH, El-Bestawy E, El-Adl NI (2004). Bioremediation of vegetable oil and grease from polluted wastewater using a sand biofilm system. World Journal of Microbiology and Biotechnology 20(6):551-557.

Er AC, Nor Abd RM, Rostam K (2011). Palm oil milling wastes and sustainable development. American Journal of Applied Sciences 8(5):436-440.

Foo KY, Hameed BH (2010). Insight into the applications of palm oil mill effluent: a renewable utilization of the industrial agricultural waste. Renewable and Sustainable Energy Reviews 14(5):1445-1452.

Grbavcic SZ, Dimitrijevic-Brankovic SI, Bezbradica DI, Siler-Marinkovic SS, Knezevic ZD (2007). Effect of fermentation conditions on lipase production by *Candida utilis*. Journal of the Serbian Chemical Society 72(8-9):757-765.

Hoog GS, Guarro J (2000). Explanatory chapters and keys to the genera, In Hoog GS, Guarro J, Gene J, Figueras MJ (eds.), Atlas of Clinical Fungi, Centraal bureau voor schimmel cultures, 2nd edn. Spain Press, Netherlands and Universitat Rovira i Virgili pp. 361-1008.

Jameel AT, Muyibi SA, Olanrewaju AA (2011). Comparative study of bioreactors used for palm oil mill effluent treatment based on chemical oxygen removal efficiencies. In: Alam MDZ, Jameel AT, Amid A (eds) Current research and development in biotechnology engineering at International Islamic University Malaysia (IIUM), 3rd edn. IIUM Press, Kuala Lumpur pp. 277-284.

Jameel AT, Olanrewaju AA (2011). Aerobic biodegradation of oil and grease in palm oil mill effluent using consortium of microorganisms. In: Alam MDZ, Jameel AT, Amid A (eds) Current research and development in biotechnology engineering at International Islamic University Malaysia (IIUM), 3rd edn. IIUM Press, Kuala Lumpur pp. 43-51.

Kirschman HD, Pomeroy R (1949). Determination of oil in oil – field waste waters. Analytical Chemistry 21(7):793-797.

Lam MK, Lee KT (2011). Renewable and sustainable bioenergies production from palm oil mill effluent (POME): win–win strategies toward better environmental protection. Biotechnology Advances 29(1):124-141.

Lan WU, Gang GE, Jinbao WAN (2009). Biodegradation of oil wastewater by free and immobilized *Yarrowia lipolytica* W29. Journal of Environmental Sciences 21(2):237-242.

Mahzad H, Sa'arib M, Mohamad AMS (2009). Optimization of POME anaerobic pond. European Journal of Scientific Research 32(4):455-459.

Maygaonkar PA, Wagh PM, Permeswaran U (2012). Biodegradation of distillery effluent by fungi. Bioscience Discovery 3(2):251-258.

Md Din, MF, Ujang Z, van Loosdrecht MCM, Ahmad A, Sairan MF (2006). Optimization of nitrogen and phosphorus limitation for better biodegradable plastic production and organic removal using single fed-batch mixed cultures and renewable resources. Water Science and Technology 53:15-20.

Mohammed RR, Ketabachi MR, McKay G (2014). Combined magnetic field and adsorption process for treatment of biologically treated palm oil mill effluent (POME). Chemical Engineering Journal 243:31-42.

Najafpour GD, Zinatizadeh A, Mohamed AR, Hasnain Isa M, Nasrollahzadeh H (2006). High-rate anaerobic digestion of palm oil mill effluent in an upflow anaerobic sludge-fixed film bioreactor. Process Biochemistry 41(2):370-379.

Nwauche CO, Ogbonna JC (2011). Isolation of lipase producing fungi from palm oil mill effluent dumpsite at Nsukka. Brazilian Archives of Biology and Technology 54(1):113-116.

Odegaar H, Ruster B, Westrum T (1998). A new moving bed biofilm reactor-application and results. Water Science and Technology 19:157-165.

Ohimain EI, Izah SC, Jenakumo N (2013a). Physicochemical and Microbial Screening of Palm Oil Mill Effluents for Amylase Production. Greener Journal of Biological Sciences 3(8):307-318.

Ohimain EI, Izah SC, Abah SO (2013b). Air quality impacts of smallholder oil palm processing in Nigeria. Journal of Environmental Protection 4:83-98.

Oswal N, Sarma PM, Zinjarde SS, Pant A (2002). Palm oil mill effluent treatment by a tropical marine yeast. Bioresource Technology 85(1):35-37.

Piro P, Carbone M, Tomei G (2011). Assessing settleability of dry and wet weather flows in an urban area serviced by combined sewer. Water Air and Soil Pollution 214(1-4):107-117.

Pogaku R, Sarbatly RH (2013). Advances in Biofuels. Springer, New York, NY, USA.

Poh PE, Chong MF (2008). Development of anaerobic digestion methods for palm oil mill effluent (POME) treatment. Bioresource Technology pp. 1-9.

Qingwei L, Mancl KM, Tuovinen OH (1998). Effect of inoculation on the biodegradation of butterfat-detergent mixtures in fixedfilm sand columns. Bioresource Technology 64(1):27-32.

Rahman RNZRA, Leow TC, Salleh AB, Basri M (2007). *Geobacillus zalihae* sp. nov., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia. BMC Microbiology 7(1):77.

Roux-Van Der Merwe MP, Badenhorst J, Britz TJ (2005). Fungal treatment of an edible-oil containing industrial effluent. World Journal of Microbiology and Biotechnology 21:947-953.

Singh NNP, Pandey A (2009). Biotechnology for Agro-Industrial-Residues-Utilisation. Vol. 1, Springer Science + Business Media B.V. New York, NY, USA.

Soleimaninanadegani M, Manshad S (2014). Enhancement of Biodegradation of Palm Oil Mill Effluents by Local Isolated Microorganisms. International Scholarly Research Notices pp.1-8.

Suseela L, Anupama M, Prudhvilal B, Narasaiah TV, Lavanya Latha JN (2014). Isolation & Characterization of lipase producing fungi from Palm Oil Mill Effluent obtained from Pedavegi, A.P., India. International Journal of Biological and Pharmaceutical Research 5(7):559-565.

Takeno K, Yamaoka Y, Sasaki K (2005). Treatment of oil-containing sewage wastewater using immobilized photosynthetic bacteria. World Journal of Microbiology and Biotechnology 21:1385-1391.

Tubonimi TKI, Adiukwu PU, Stanley HO, Briggs AO (2007). Impact of Palm oil (*Elaeis guineensis Jacq; Banga*) Mill Effluent on Water Quality of Receiving Oloya Lake, in Niger Delta, Nigeria. Research Journal of Applied Sciences 2(7):842-845.

Vijayaraghavan K, Ahmad D, Abdul Aziz ME (2007). Aerobic treatment of palm oil mill effluent. Journal of Environmental Management 82(1):24-31.

Wakelin NG, Forster CF (1997). An investigation into microbial removal of fats, oils and greases. Bioresource Technology 59:37-43.

Wu TY, Mohammad AW, Jahim JM, Anuar N (2009). Optimized reuse and bioconversion from retentate of pre-filtered palm oil mill effluent (POME) into microbial protease by *Aspergillus terreus* using response surface methodology. Journal of Chemical Technology and Biotechnology 84:1390-1396.

Wu TY, Mohammad AW, Jahim JM, Anuar N (2010). Pollution control technologies for the treatment of palm oil mill effluent (POME) through end-of-pipe processes. Journal of Environmental Management 91:1467-1490.

African Journal of Biotechnology

Full Length Research Paper

Variation of concentrations of thymol and carvacrol in the essential oil of thymus satureoides during aging

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The aim of this work is to do a stability study of two chemo-typical active ingredients (thymol and carvacrol) of the essential oil of thyme "*Thymus satureoides*" collected from Azilal Region, Morocco. Specimen identification was performed for the raw obtained plant materials and the essential oil extraction carried out through initial and final chemical characterization of the essential oil - at t = 0 - and - at t = 6 months. Results show that a significant decrease (13%) in the initial thymol concentration was recorded during the assumed duration of the stability study (6 months). Carvacrol recorded an even larger drop and was approaching 25%, during the same study period.

Key words: Stability, concentration, essential oil, *thymus satureoides*, thymol, carvacrol.

INTRODUCTION

Thyme has more than 200 varieties with an endemism of about 46.6% in Morocco (Fennane et al., 2007). In Morocco, the genus *Thymus* (Lamiaceae) is represented by 21 species of which 12 are endemic (Benabid, 2000). The essential oil of thyme has various pharmacological properties, including antibacterial (Boscovic et al., 2017; Chraibi et al., 2016), antifungal (Ben et al., 2017; Jamali et al., 2012), and anti-oxidant properties (Tohidi et al., 2017; Kasrati et al., 2015; Jamali et al., 2012).

The practical study focuses on the analysis of the degree of conservation of the active principle (s), especially the two chemo-typical active ingredients of the plant (thymol and carvacrol) as well as the study and analysis of the variations of their rates and concentrations during the duration of their conservation. This relates to the stability and therefore the pharmacological, toxicological and ecological properties of the "essential oil".

MATERIALS AND METHODS

Thyme satureioïdes - flowering aerial parts were collected at the zone level: Bin Elouidane, Azilal / Beni Mellal Province, Morocco, with the help of local collectors. The specimen was authenticated before and after harvesting according to the vascular plants of Morocco - Practical Flora of Morocco (Fennane et al., 2007) where a representative sample of the plant was deposited at the Scientific Institute of Rabat - Department of Herbal Botany and Ecology, 15

Table 1. Representation of injection conditions.

Injection volume	1 µl
Injection temperature	220°C
Interface temperature	300°C
Injection mode	SPLIT
Vector gas	Hélium
Flow rate	1,4ml/min

June, 2016, for official botanical identification.

Our working sample was officially identified by Dr. El oualidi Jalal, Head of the Department of Botany and Plant Ecology, Rabat Scientific Institute as *T. satureoides;* it is referred to as "*T. saturioides* Cosson in Bull. Soc. Bot. France 58: 436. 1911 AS AA MA MA Mam -2-3"and registered at the herbarium of the same institute with the index number: RAB98065.

One hundred grams flowering aerial parts of thyme in the semi-fresh state (only 5 days of drying in the open air and at room temperature) were used. The extraction was done in June 16th and 17th, 2016 at the laboratory of the Faculty of Medicine and Pharmacy of Rabat. A Clevenger extraction system consisting essentially of a glass balloon with a capacity of 2 L surmounted by a column and a condenser was used. This was done in accordance with the internal procedure of the laboratory relating to the extraction of the essential oil from the flowering aerial parts of thyme by academic hydro-distillation. The quantity of essential oil of *T. satureioides* (EO / TS) recovered was put in vials, using a sterile syringe, as no specific method of preservation (such as sub vacuum or under nitrogen) was considered in this case study. The two "vials" or small bottles containing the essential oil are kept separately. The first vial is stored at a temperature of 4°C in the dark in order to preserve the initial composition of the essential oil and the second vial was placed in a climatic chamber under conditions of accelerated aging; temperature of 40°C and a relative humidity of 75% for a period of 6 months.

Initial chemical characterization of the essential oil EO / TS at t = 0

A first analysis (at t = 0) of the chemical composition of EO / TS was carried out at the National Center for Scientific and Technical Research "CNRST" / Division of Technical Support Units for Research Scientific "UATRS" - Rabat - Morocco, under the following conditions:

GPC / MS Analysis Conditions = UATRS Standard Conditions (Essential Oil)
Apparatus: Gas chromatograph (TRACE GC ULTRA) coupled to a mass spectrometer (Polaris Q MS with ion trap).
Type of analysis performed: Qualitative and quantitative.
Type of ionization: Electronic impact (70 eV)
Solvent type: n-Hexane or ethyl acetate
Column type: VB-5 (Methylpolysiloxane 5% phenyl), 30 m * 0.25 mm * 0.25 µm.
Injection conditions: Table 1
Separation conditions: Table 2

Data processing

The data obtained after the analysis are processed in a computer,

in the form of digital data. This allows the development of a spectrogram which is a graphical presentation of the spectral map (peaks) of the various components of the analyzed product. Special software, integrated into the GC-MS system, generally handles the acquisition and processing of digital data from the detector, while at the same time it can be interpreted by automatic comparative study with reference scientific data contained in laboratory-specific computerized libraries.

Final chemical characterization (in post - aging) of the essential oil extract E O/ T.S at t = 6 months

The essential oil sub-sample having undergone the above-mentioned conditions of accelerated aging was subjected to a final analysis of its chemical composition following the same procedure (analysis and treatment of results) mentioned above. **Comparing the individual areas of each substance separately (thymol and carvacrol), at t = 0 and t = 6 months and calculating the percentage difference**

Whereas all the parameters of the analysis (GC / MS) of all the samples at t = 0 and at t = 6 months are fixed and identical, particularly, those linked to the technique of analysis, equipment used, manipulation of the analyst, analytical environment (temperature, humidity, pressure ...) and the automatic interpretation of the software. We applied a very simple approach of calculation, by direct comparison of the individual areas corresponding to each substance separately, successively obtained at t = 0 and at t = 6 months. The calculation was done as follows:

For carvacrol
A_C t0: carvacrol Area at t = 0.
A_C t6: Carvacrol area at t = 6 months.
Whereas A_C t0 corresponds to 100% of the substance.
Calculate the percentage (X) of A_C t6 compared to A_C t0, through the equation (rule of three):
$X = A_C$ t6 / A_C t0 x 100%
Calculate afterwards the difference "E_C" (in percentage) between A_C t0 and A_C t6:
E_C = 100% - X

For the thymol
A_T t0: Thymol area at t = 0.
A_T t6: Thymol area at t = 6 months.
Whereas A_T t0 corresponds to 100% of the substance.
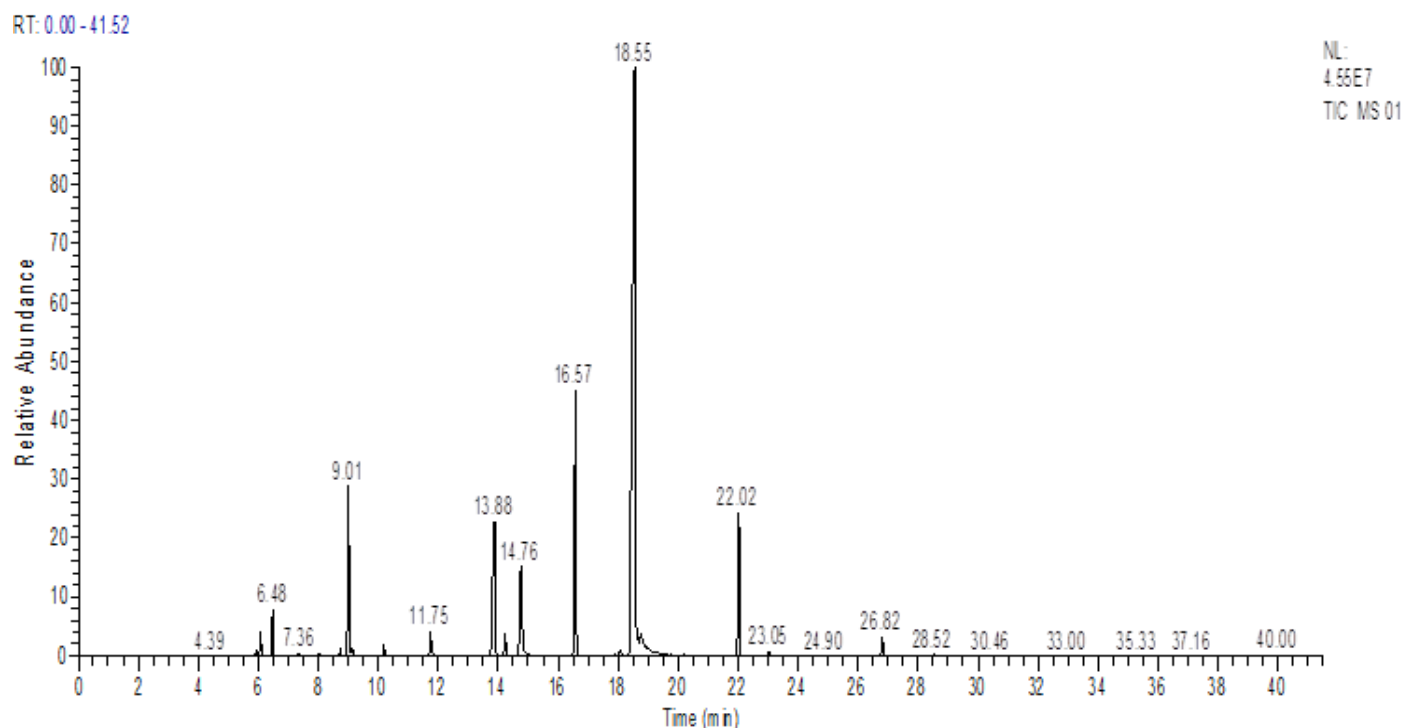Calculate the percentage (Y) of A_T t6 compared to A_T t0, through the equation (rule of three):
$Y = A_T$ t6 / A_T t0 x 100%
Calculate, afterwards, the difference "E_T" (in percentage) between A_T t0 and A_T t6:
E_T = 100% - Y

Table 2. Representation of separation conditions.

Ramp (°C /min)	Final Température (°C)	Time (min)
	40	2.00
4	180	0.00
20	300	2 .00

Figure 1. Initial spectrogram (graphic presentation of the spectral map (peaks)) of the different components of *T. satureoides* essential oil at t = 0. Retention times: RT = 16.57 and RT = 18.55 correspond successively to "Carvacrol" and "Thymol".
Source: Elkacimi I. (2016).

RESULTS

Extraction step

Approximately 2.5 ml (2.22 g) of *T. saturioides* essential oil (EO / TS) was extracted from almost 100 g of the raw material for two days (at the rate of 1.5 ml for the first day and 1 ml for the second day) in the laboratory of the Faculty and in accordance with the above academic procedure.

Initial analysis step: initial chemical characterization (at t = 0) of the essential oil E.O / T.S.

The result of initial qualitative analysis (at t = 0) of the studied essential oil using gas chromatography coupled with mass spectrometry (GC / MS) is summarized in the

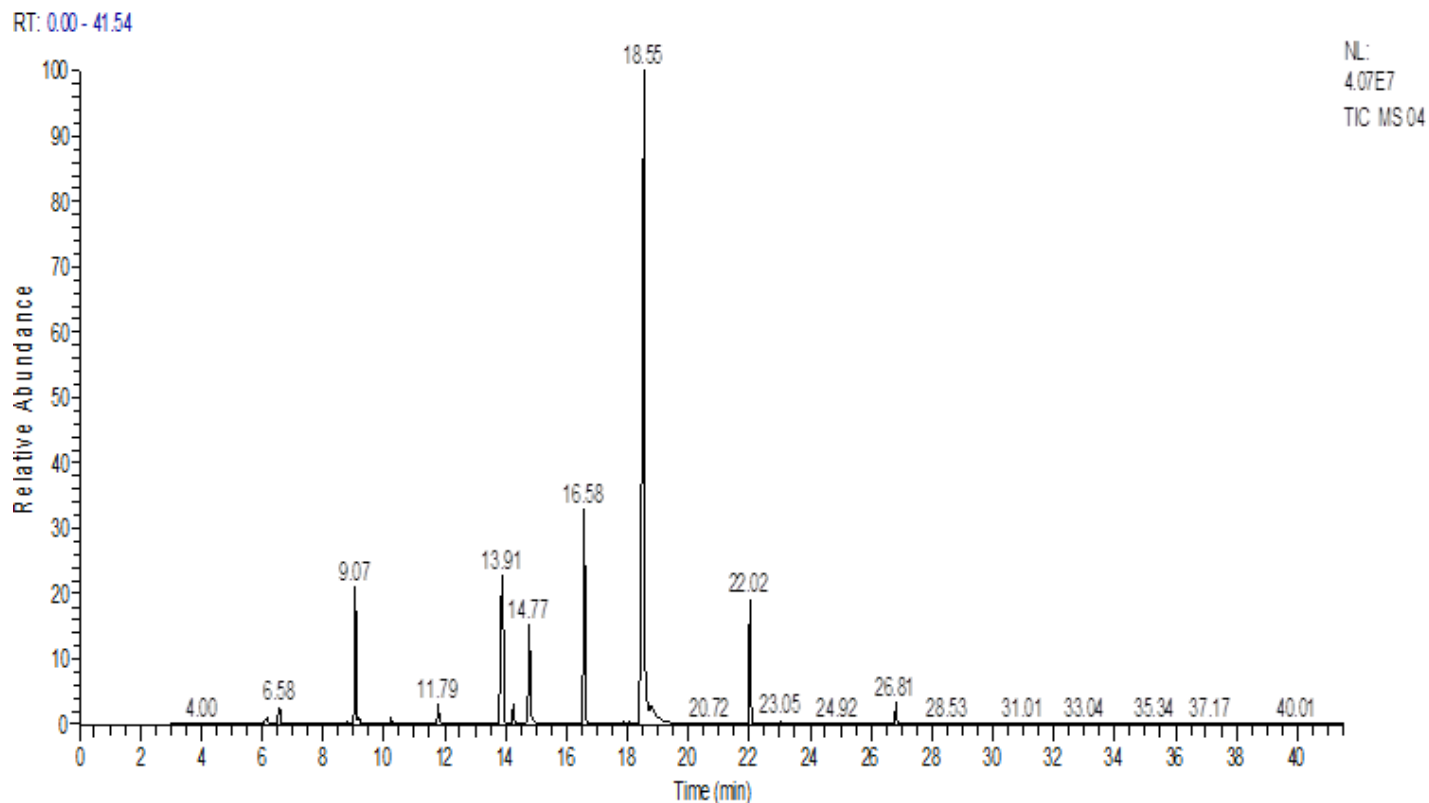Figure 1.

Final analysis step: Final chemical characterization (in post-aging) of the essential oil extract E.O / T.S

The result of the final qualitative analysis (at t = 6 months after aging under: t = 40 ° C and HR = 75%) of the essential oil using gas chromatography coupled with mass spectrometry (GC / MS) is summarized in Figure 2.

Calculation of individual percentages and deviations

The quantitative results of the two main components (carvacrol and thymol) are listed in Table 3. These results are in accordance with the following basic principles of calculation:

Figure 2. Spectrogram (graphic presentation of the spectral map (peaks)) of the various components of the saturated thyme essential oil at t = 6 months after aging under: t = 40°C and HR = 75%. Retention times: RT = 16.58 and RT = 18.55 correspond successively to "Carvacrol" and "Thymol".
Source: Elkacimi I. (2016).

Table 3. Representation of the results of the quantitative analysis (at t = 6 months after aging under: t = 40 ° C and HR = 75%) of carvacrol and thymol compared to the initial composition (100%) of the same substances in essential oil of *T. satureoides* (EO / TS).

Retention time (RT)	Substance	Individual percentage of the substance at t = 6 months relative to its initial concentration at t = 0	Difference (in percentage) between the concentration of the same substance between t = 0 and t = 6 months
T = 0 / t = 6 months 16.57 / 16.58	Carvacrol	76.51%	23.49%
T = 0 et t = 6 months 18.55	Thymol	86.94%	13.06%

Source: Elkacimi I. (2016).

For carvacrol

A_C t0: Carvacrol area (at t = 0) = 67097757
A_C t6: Carvacrol area (at t = 6 months) = 51339704
Whereas A_C t0 corresponds to 100% of the substance.
Calculation of the percentage (X) of A_C t6 with respect to A_C t0, through the equation (rule of three):
X = A_C t6 / A_C t0 x 100% = 51339704/67097757 x 100% = 76.51%

Calculation, afterwards, of the difference "E_C" (in percentage) between A_C t0 and A_C t6:
E_C = 100% - X = 100 - 76.51% = 23.49%

For the thymol

A_T t0: Thymol area (at t = 0) = 302397036
A_T t6: Thymol area (at t = 6 months) = 262920106
Whereas A_T t0 corresponds to 100% of the substance.

Calculation of the percentage (Y) of AT t6 with respect to AT t0, through the equation (rule of three):

Y = AT t6 / AT t0 x 100% = 262920106/302397036 x100% = 86.94% Calculation, afterwards, of the difference "ET" (in percentage) between AT t0 and AT t6:

E_T = 100% - Y = 100 - 8. 94% = 13.06%

DISCUSSION

In the first place and according to spectrograms (Figures 1 and 2) resulting from the GC / MS analysis of the essential oil (EO / TS), the specific identity of this variety of thyme can be confirmed. Because the concentrations of both thymol and carvacrol are more compared to those of other components including that of "borneol", which is not the case of the variety of the plant generally known by its chemotype rich in borneol, it is therefore deduced that it is a second (non-ordinary) variety of saturated thyme as reported by Benjilali et al. (1987). This hypothesis is verified by other research carried out on essential oils of T. satureioides (EO / TS) from different regions of Morocco. This is the case of EO / TS, from Ifrane Region, whose major component is "P-cymene" (Elouali et al., 2013) or the case of EO / TS from Taroudant Region that is rich in "borneol" (Ramzi et al., 2017). For the EO / TS essential oil extraction, it has a total yield of about 2.22%, with more than 1.1% obtained by Elouali et al. (2013).

On the other hand, by making a comparative study of the quantitative results relating to the levels of the two components (thymol and carvacrol) obtained following the stability study of the essential oil (between t = 0 and t = 6 months under accelerated aging), we found a significant decrease of more than 13% in the initial thymol concentration over the assumed duration (6 months), from 100% at t = 0 to 86.94% at t = 6 months. Carvacrol decreased even more, approaching 25% over the same study period, from 100% to t = 0 to 76.51%. t = 6 months.

It can be deduced that, in general terms, this decrease, no matter how large, in the concentration of the two substances during the study period (6 months) may be essentially due to their degradation under the sudden climatic and environmental factors (mainly redox reactions) effects on one hand and, on the other hand, to the use of the essential oil as it is (traditional preservation) without being subject to any industrial preservation (under vacuum or under nitrogen). It should also be noted that the degradation is greater in the case of carvacrol with more than 23% compared to that of thymol (13%). This led us to suggest that carvacrol may be more sensitive to degradation factors than thymol with a difference of more than 10%.

Conclusion

From the foregoing, it can be concluded that the essential oil of T. satureioïdes (HE / TS) is subject to rapid deterioration in the case of artisanal conservation, in time and under the effect of climate degradation factors. This encourages us to recommend complementary studies in the same direction, so as to include, among others:

Stability study of vacuum conditioned EO / TS.
The stability study of the EO / TS conditioned under nitrogen.
The stability study of the EO / TS added conservator (s).

This will serve to form a general and more precise idea of the stability status of the thyme essential oil (EO / TS) for each type of conditioning and preservation and especially, for comparative study using traditional packaging and preservation, which is the approach adopted in this study. The ultimate goal sought here is to find, with tangible evidence to support, the best way to keep the essential oil of T. satureoides. This could be valid for other essential oils.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Benabid A (2000). Flore et écosystèmes du Maroc : Evaluation et préservation de la Biodiversité.Ibis Press & Editions Kalila Wa Dimna. 360 p.

Ben Jabeur M, Somai-Jemmali L, Hamada W (2017). Thyme essential oil as an alternative mechanism: biofungicide-causing sensitivity of *Mycosphaerella graminicola*. Journal of Applied Microbiology 122(4):932-939.

Boskovic M, Djordievic J, Ivanovic J, Janjic J, Zdravkovic N, Gliscic M, Glamoclija N, Baltic B, Djordjevic V, Baltic M (2017). Inhibition of *Salmonella* by thyme essential oil and its effect on microbiological and sensory properties of minced pork meat packaged under vacuum and modified atmosphere. International Journal of Food Microbiology 258:58-67.

Chraibi M, Farah A, Lebrazi S, El Amin O, Iraqui HM, Fikri-Benbrahim K (2016). Antimycobacterial natural products from Moroccan medicinal plants: Chemical composition, bacteriostatic and bactericidal profile of *Thymus satureioides* and *Mentha pulegium* essential oils. Asian Pacific Journal of Tropical Biomedicine 6(10):836-840.

Elouali LA, El akhal F, Ouedrhiri W, Ouazzani CF, Guemmouh R, Greche H (2013). Composition chimique et activité antibactérienne des huiles essentielles de deux plantes aromatiques du centre nord marocain: Thymus vulagris et *Thymus satureioïdis*. Les technologies de laboratoire 6:27-33.

Fennane M, Ibn tattou M, Ouyahya A, Eloualidi J (2007). Flore pratique du Maroc: Manuel de détermination des plantes vasculaires du Maroc. Institut Scientifique de Rabat 2:648.

Ramzi H, Ismaili MR, Aberchane M, Zaanoun S. (2017). Chemical characterization and acaricidal activity of *Thymus satureioides* C. & B. and *Origanum elongatum* E. & M. (*Lamiaceae*) essential oils against *Varroa destructor* Anderson & Trueman (*Acari*: *Varroidae*). Industrial Crops & Products 108:201-207.

Jamali CA , El Bouzidi L, Bekkouche K, Lahcen H, Markouk M, Wohlmuth H, Leach D, Abbad A (2012). Chemical Composition and Antioxidant and Anticandidal Activities of Essential Oils from Different Wild Moroccan *Thymus* Species. Chemistry & Biodiversity 9(6)1188-1197.

Kasrati A, Jamali AC, Bekkouche K, Wohlmuth H, Leach D, Abbad A (2015). Comparative evaluation of antioxidant and insecticidal properties of essential oils from five Moroccan aromatic herbs. Journal of Food Science and Technology 52(4):2312–2319.

Tohidi B, Rahimmalek M, Arzani A (2017). Essential oil composition, total phenolic, flavonoid contents, and antioxidant activity of Thymus species collected from different regions of Iran. Food Chemistry220: 153-161.

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