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Full Length Research Paper

Karyotype analysis of obtained tetraploid in medicinal plant (*Platycodon grandiflorus*)

Fuhong Yang[1], Lingjiao Li[2], Erhua Rong[2], Yongbo He[3], Xiaoming Zhao[2] and Yuxiang Wu[2]*

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***Platycodon grandiflorus* is an important medicinal plant in China. Induction of polyploidy was successfully attempted in this medicinal plant using 0.05% colchicine semi-solid under 72 h by apical shoot tip treatment of young seedlings, and then 50% mutant plants were obtained. It was testified by the chromosome number in mitosis and its karyotype analysis for diploid control and obtained mutants. The results showed that the chromosome number of the diploid plant was 2n=2x=18, whereas the chromosome number of the obtained mutants was 2n=4x=36. The karyotype formula of mutant plant and diploid control were K=2n=4X=36=24m+12sm (4SAT) and K=2n=2X=18=12m+6sm (2SAT), respectively by karyotype analysis and they belonged to 2B and 2A karyotype. It was confirmed that the obtained mutant plants were tetraploid.**

Key words: *Platycodon grandiflorus*, Obtained mutants, karyotype analysis.

INTRODUCTION

Platycodon grandiflorus, a species of perennial flowering plant of the family Campanulaceae, is native to East Asia (such as China, Korea, Japan, and East Siberia) and has been used as an important medicine or food for centuries in China. In Europe, it is cultivated mainly as an ornamental plant (Lee et al., 2002). However, its potential health care uses have become a hot research topic in Asia. As far as in 1970s, the root of this species (radix) was extensively used as an anti-inflammatory in the treatment of coughs and colds (Lee, 1973); a finding of Jang et al. (2013) suggested this effect in lipopolysaccharide stimulated BV2 microglial cells. In addition, according to some recent reports, saponins from the roots of *P. grandiflorus* (CKS), as one of the most essential functional components in radix, have been shown to exhibit many other pharmacological activities, including anti-cancer (Park et al., 2005; Lee et al., 2004, 2013; Jeong et al., 2010; Chun et al., 2013), antioxidant (Jeong et al., 2010; Ryu et al., 2012), the inhibition of pancreatic lipase (Zhao and Kim, 2004), anti-allergic (Han et al., 2009), anti-hyperglycemiccholesterol (Ahn et al., 2012; Jang et al., 2010), anti-skin photoaging (Hwang et al., 2011), stimulate osteoblast differentiation (Jeong et al., 2010), lowering Cholesterol (Zhao, 2006), and anti-obesity effects (Zhao et al., 2006; Hwang et al., 2013).

Moreover, another component of root, named

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homogalacturonan, was found having a property of anti-angiogenesis activity (Xu et al., 2011), suggesting its potential to be developed as an anti-angiogenesis drug. Chen et al. (2013) also demonstrated the intervention of effective parts extracted from *P. grandiflorus* on microangiopathy of diabetic rats. Hence, *P. grandiflorus* has gradually developed into an effective remedy used in many kinds of medicines as a treatment for some intractable diseases, such as cancer, glycuresis, obesity, airway diseases (Choi et al., 2011), which is no longer restricted to traditional ills like cough. Due to its increased demand for this medicinal material, it is necessary to focus on its breeding program.

So far, most researchers are much interested in collecting wild germplasm *P. grandiflorus*, resulting in the lack of natural resources. In order to keep sustainable state, polyploid breeding make it possible to increase new germplasm in this medicinal plant. In our former study, induction of polyploidy was attempted using 1% agar with 0.05% colchicine semi-solid under 72 h by apical shoot tip treatment of young seedlings. A single drop (2-4 µl) of the warm (~50°C) semi-solid was painted between cotyledons of each seedling to cover the apical bud. That was one application and three applications were conducted in every 24 h interval time for totally three times. Finally, 50% mutant plants were successfully obtained in *P. grandiflorus* by preliminary morphology identification (Wu et al., 2011). In this study, the chromosome number was counted in mitosis and comparative karyotype analysis was measured by the induced mutants and diploid control. The purpose was to further check its ploidy level for induced mutants and to determine its karyotype characteristics as compared with its diploid control.

MATERIALS AND METHODS

Plant

P. grandiflorus came from Natural Medicinal Plant Garden in Shanxi province. Field experiments were finished in this garden and inside experiments were carried out in Herbal Breeding Laboratory of Shanxi Agricultural University.

Mitotic karyotype analysis

For Karyology, seeds of the obtained mutants and diploid controls were collected and germinated on moist filter paper lining petri dish at approximately 27°C. 0.5 to 1 cm long freshly grown root tips were cut off at 8 to 9 am to obtain mitotic metaphases and determine karyotype characteristics, including chromosome numbers, chromosome length and total length of all chromosomes. Firstly, these root tips for pretreatment were placed into a saturated solution of p-Dichlorobenzene and aqueous α-bromonaphthalene for 2.5 h at 20°C, and then fixed in 3:1 v/v absolute ethanol:glacial acetic acid for a minimum of 24 h at 4°C. The fixed tips were then washed thoroughly in distilled water and meristems were hydrolysed in 1 M hydrogen chloride (HCl) for about 2 min at room temperature. 1 to 2 mm length from the tips were cut and placed on clear glass slides with Carbol fuchsin solution as the staining. Squash technique was made for cytological studies. These scattered cells were were photographed through a 10×40 microscope with an OLYMPUS BX51 digital camera. 10 best metaphases were selected for further measures by Photoshop 8.0 software to study the karyotype. The method for karyotypic analysis followed with Li and Chen (1985) and the karyotype classification was based on Stebbins (1971).

RESULTS

Chromosome numbers analysis

Both the numbers and the characters of chromosomes in mitosis are the most persuasive checking standards for the ploidy level. In this research, karyotypic characters, mitotic metaphase chromosomes of diploid control and the mutants are as shown in Tables 1 and 2 and Figures 1 to 4, respectively. Analysis of somatic metaphases showed that the chromosome numbers of mutants were 2n=4x=36, while the chromosome numbers of diploid control were 2n=2x=18. It is the direct evidence for this polyploid induction in *P. grandiflorus*.

Karyotype analysis of diploid control

Statistical observations were made for more than fifty root tip cells, all of which were at mitotic metaphase and could be counted for the chromosome number. The metaphase chromosomes of the diploid *P. grandiflorus* are as shown in Figure 1. The relative length of chromosomes in the diploid control was from 4.18 to 7.67. Arm ratios ranged from 1.08 to 2.00 and the average arm ratio was 1.481. The karyotypic formula was 2n=2x=18=12m+6sm (2SAT), consisting of 12 metacentric chromosomes and 3 pairs of submetacentric chromosomes (Table 1), and a satellite on the short arm of group VI chromosomes was observed. The karyotype was type 2A (Figure 2).

Karyotype analysis of obtained mutants

More than fifty root tip cells were statistically observed, all of these cells were at mitotic metaphase and could be counted the chromosome numbers for obtained mutants. The metaphase chromosomes of the mutants *P. grandiflorus*, which are as shown in Figure 3, were arranged into 9 groups of four chromosomes each. The chromosomes in each group had a similar morphology. Relative lengths ranged from 2.02 to 4.05, and arm ratios ranged from 1.18 to 2.45. The average arm ratio was 1.622 (Table 2). Four satellites were seen on the short arms of the eleventh and twelfth chromosomes (Figure 4). The karyotype was type 2B (Table 2) and the karyotypic formula was 2n=4x=36=24m+12sm (4SAT).

DISCUSSION

Our study has illuminated the karyotype characters of the medicinal plant *P. grandiflorus* through comparative karyotype analysis of the diploids and induced mutants, while demonstrating the previous study (Wu et al., 2011; Zhao et al., 2004) in which the somatic chromosome number of diploid *P. grandiflorus* was noted as 2n=2x=18 and the chromosome number of obtained tetraploids was 2n=4x=36, both of which with basic chromosome number x=9 by chromosome counting in root-tip cells.

According to Stebbins (1971), karyotype evolution is generally from symmetry to asymmetry in higher plants

Table 1. The coefficient of chromosomes of diploid *P. grandiflorus*.

Number	Relative length (%)			Arm ratio (Long/Short)	Type
	Long	Short	Total length		
1	5.08	2.59	7.67	1.96	sm
2	4.78	2.39	7.17	2.00	sm
3	3.98	2.29	6.27	1.74	sm
4	2.99	2.59	5.58	1.154	m
5	2.59	2.39	4.98	1.08	m
6	2.79	1.99	4.78	1.40	M*
7	2.39	2.19	4.58	1.09	m
8	2.59	1.79	4.38	1.44	m
9	2.49	1.69	4.18	1.47	m

*Sat-chromosome. The length of satellites is not included in the chromosome length. Chromosome number: 2n=2x=18. Karyotype formula: 12m＋6sm(2SAT). Karyotype Type: 2A, RATIO of chromosome length (L/S): 1.83. Proportion of chromosome of arm ratio>2: 0.11.

Table 2. The coefficient of chromosomes of obtained tetraploid *P. grandiflorus*.

Number	Relative Length (%)			Arm ratio (Long/Short)	Type
	Long	Short	Total length		
1	2.76	1.29	4.05	2.14	Sm
2	2.76	1.29	4.05	2.14	sm
3	2.48	1.01	3.49	2.45	Sm
4	2.30	1.10	3.40	2.08	sm
5	1.84	1.56	3.40	1.18	m
6	1.84	1.56	3.40	1.18	m
7	1.75	1.10	2.85	1.58	m
8	1.66	1.10	2.76	1.50	m
9	1.66	1.01	2.67	1.64	m
10	1.66	1.01	2.67	1.64	m
11	1.56	0.92	2.48	1.70	m*
12	1.47	1.01	2.48	1.45	m*
13	1.19	1.01	2.20	1.18	m
14	1.19	1.01	2.20	1.18	m
15	1.38	0.74	2.12	1.88	sm
16	1.38	0.74	2.12	1.88	sm
17	1.10	0.92	2.02	1.20	m
18	1.10	0.92	2.02	1.20	m

*Sat-chromosome. The length of satellites is not included in the chromosome length. Chromosome Number: 2n=4x=36. Karyotype formula: 24m＋12sm(4SAT). Karyotype Type: 2B, Ratio of chromosome length (L/S): 2.00. Proportion of chromosome of arm ratio>2: 0.22.

generally from symmetry to asymmetry in higher plants during the biological evolution process. Karyotypes are classified into 12 types (1A-4A, 1B-4B, 1C-4C) based on analysis of plant materials. This theory suggests that primitive plants have symmetric karyotypes whereas asymmetric karyotypes occur in more evolutionary advanced taxa. In this study, the karyotype of the tetraploid showed that the ratio of the longest chromosome to the shortest chromosome was 2.00 and the percentage of chromosome with arm ratio exceeding 2 was 22%. It belonged to the karyotype of 2B, which was consistent with the result by Wang et al. (2006b). While the diploid control belonged to 2A in this study and followed with the result (Wang et al., 2006a). This indicated that the intrachromosomal symmetry in the diploid was higher than in the tetraploids. In addition, Wang et al.

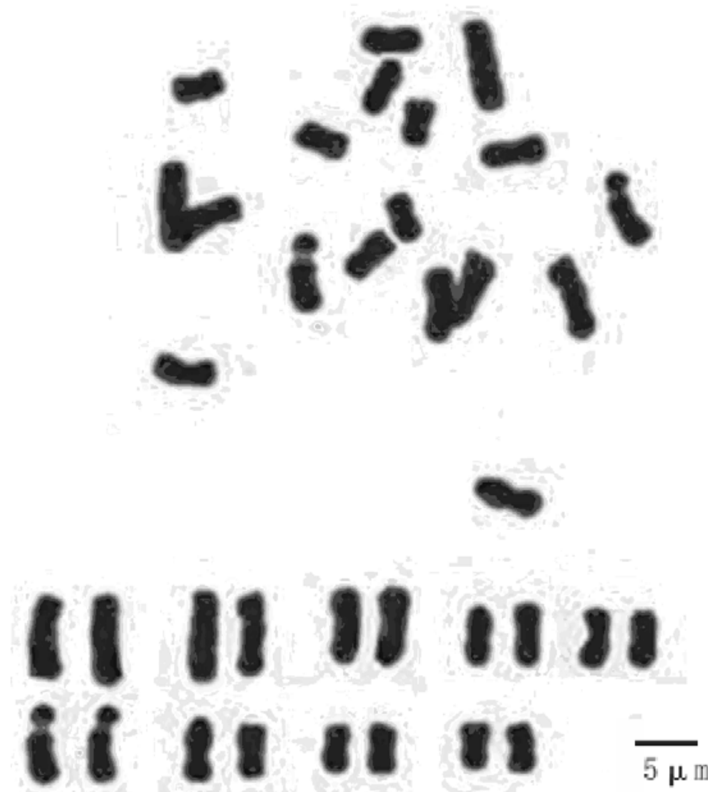

Figure 1. The morphology of somatic chromosomes of diploid *P. grandiflorus*.

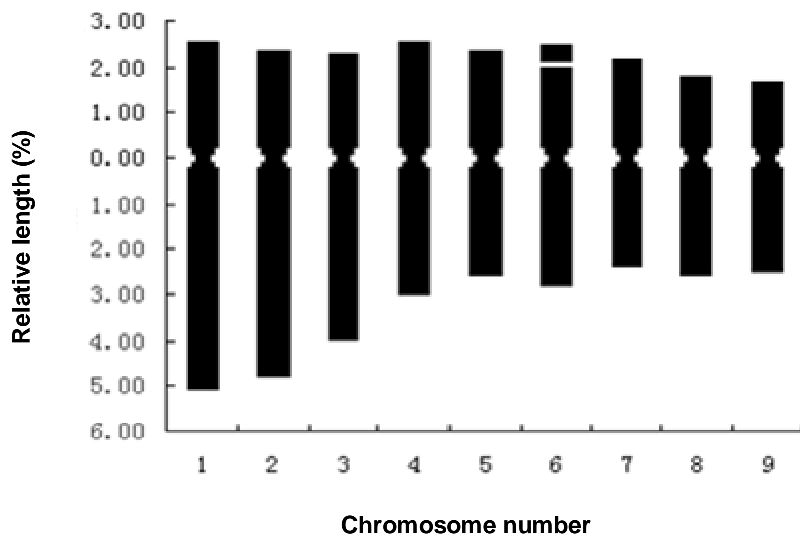

Figure 2. The karyotype of diploid *P. grandiflorus*.

(2007) found a haploid karyotype of *P. grandiflorus* belonging to the type 1A, exhibited higher symmetric and primitive. This suggested that the tetraploids were advanced than its other types with low ploidy level. This phenomenon showed that a plant with the higher ploidy level may be advanced than those with lower ploidy levels. From this study we also confirmed that the tetra-ploid genome got rearranged and its fragments may be

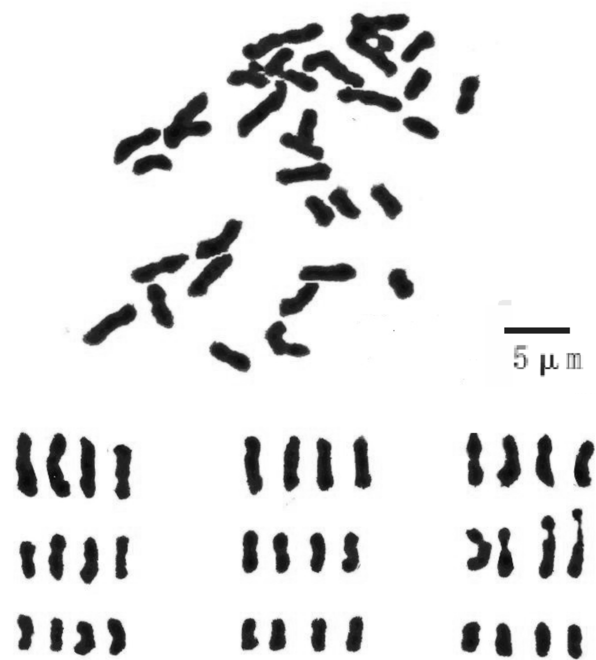

Figure 3. The morphology of somatic chromosomes of tetraploid *P. grandiflorus*.

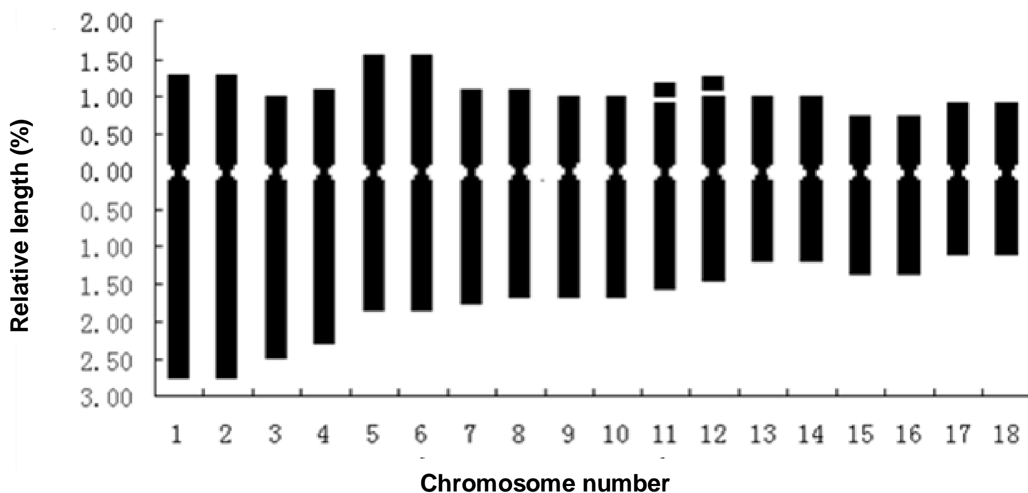

Figure 4. The karyotype of obtained mutants *P. grandiflorus*.

maybe get lost (Desai, 2006).

In the present study, octoploid (8x), mixoploid (2x + 4x), except diploid and tetraploid in some shoot-tip meristem cells were found. This has been caused by abnormal mitosis or meiosis induced by doubling chromosome number, as was expected, it led to some abnormal phenomenon during the meiosis of its PMCs, such as quadrivalent, laggard chromosomes and irregular chromosome bridges (Wu et al., 2012). High-level polyploids can be stunted or infertile, possibly resulting from the extreme genetic redundancy and somatic instability that leads to chimera tissue (Ockendon, 2008). In this study, the occurrence of mixoploidy was compared between the second generations M_2 and M_1, and found that the proportion of chimera was lower in the second generation M_2 with about 3% than M_1 in *P. grandiflorus*. In short, the results of the present work have improved the understanding of the organization, structure, and

evolution of the genome of *P. grandiflorus*. Different types of polyploidy levels, especially autotetraploidy of this medicinal plant will provide abundant germplasm resources for treatments of diseases. Polyploid plant may have much more functional components in its vegetative organs and its function will be much improved. There will be continuation in the report of changes of the components of polyploidy plant and its new functions in later study.

Conclusions

From this study, mutant plants of *P. grandiflorus* obtained in our former study was further identified as tetraploids by its 36 chromosomes and its karyotype formula K=2n=4X=36=24m+12sm (4SAT) as compared to its diploid control with 18 chromosomes and karyotype formula K=2n=2X=18=12m+6sm (2SAT). They belonged to 2B and 2A karyotype, respectively.

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Conflict of interest

Authors have not declared any conflict of interest.

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Full Length Research Paper

Comparison of allopathic and herbal medicine for the treatment of *Entamoeba histolytica*: A double blind clinical trial

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This parallel, randomized, double blind clinical trial was designed to compare allopathic drugs [a combination of metronidazole + diloxanide furoate (MDF)] with Endemali (herbal product) for therapeutic cure rate and side effects in order to find out the most suitable drug for this killer disease. This double blind randomized clinical trial was conducted in two areas of Karachi, Pakistan after approval from ethical committee of Hamdard University. All those confirmed for *Entamoeba histolytica* were included in the study. One hundred and seventy one patients selected for the study were randomly allocated to two arms of 86 and 85 for allopathic and herbal treatment, respectively. However 78 in allopathic and 75 in herbal group completed the study. Main outcome variable was treatment success or failure. Secondary outcome measures included side effects and association with age and sex. No significant difference was observed in the socio economic and demographic variables at the baseline. No significant difference was found between the cure rate of MDF and Endemali; hence both drugs were equally effective in treating amoebiasis. Significant differences were reported for the side effects observed among the two groups and the price. The failure rate for the two drugs was 28.7%. It is concluded that both Endemali and MDF are equally effective in treating amoebiasis. However, Endemali has fewer side effects than MDF. New drugs need to be researched for the treatment of *E. histolytica* because of high failure rate of the two drugs against this killer organism.

Key words: Comparison, *Entamoeba histolytica*, amoebiasis, herbal, Endemali, metronidazole, diloxanide furoate, clinical trial, double blind, parallel.

INTRODUCTION

Human intestinal pathogenic parasites (HIPP), especially *Entamoeba histolytica* have significant effect on the health

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of the community (Mondal, 2006; Herbinger, 2011; Siddiqui, 2002). In highly uneducated societies, like Pakistan, where literacy rate is 34% and only 20% population has access to safe water supply and sanitation and even lesser in the rural area, the prevalence of parasitic infection is a common problem (Iliyas, 2006) which requires effective treatment. Many options exist for the treatment of parasitic infection including herbal medicine, which are said to be more cost effective with less side effects (Dans, 2007; Shahab-ud-din, 2006; Al-Habbal, 1984; Calzada, 2005). Some work has been done regarding the prevalence of HIPP in Pakistan but there were few studies conducted to compare allopathic and herbal modalities for the treatment of amoebiasis (Khan, 2008; Ranque, 2004). Various options are available for the treatment of amoebiasis with a variable degree of failure rate (Dans, 2007) and include metronidazole, secnidazole, etc.

Huston (2003) conducted a randomized double blind study and found that natural herbal medicine showed significantly better result than placebo with a p-value < 0.04. One of the laboratories in Pakistan has also developed certain herbal medicine under the Unani pharmacopeia including Endemali to treat amoebiasis which has been tested for years for its efficacy and safety (Shahab-ud-din, 2006; Ozcan, 2004).

This double blind clinical trial was designed to compare allopathic drug metronidazole + diloxanide furoate (MDF) with the herbal medicine, Endemali to evaluate therapeutic cure rate and side effects in order to find out the most suitable drug for this killer disease.

Objectives of the study

1. To find out the cure rate of MDF and Endemali.
2. To record the side effects reported by the two drugs.
3. To compare two drugs for efficacy and side effects.

Null hypothesis

There is no difference in the efficacy and side effects of allopathic choice of MDF (metronidazole + diloxanide furoate) and herbal product Endemali for the treatment of *E. histolytica*.

Alternate hypothesis

There is some difference in the efficacy and side effects of allopathic choice of metronidazole + diloxanide furoate (MDF) and herbal product Endemali for the treatment of *E. histolytica* (two tail hypotheses).

MATERIALS AND METHODS

Study design

It was a parallel randomized double blind clinical trial to evaluate these drug's effect by statistical analysis. Both physicians and patients were blinded about the type of treatment. Consolidated standards of reporting trials (CONSORT) flow chart and check list were used to write the methodology.

Sample population

Two areas, one in Shifa-Ul-Maluk Hospital, situated in the rural setting of Ali Goth, Gadap, Karachi and the other in Zahida Medical Centre, Sector 5-C, North Karachi were selected to randomly allocate the patients in two groups that is, allopathic group and herbal group.

Sample size

To determine the sample size, we used $\alpha < 0.05$ and power of study as 90%. The effect size considered to be clinically significant was a 0.15 difference between two groups based on a pilot study. "Hypothesis test for two population proportion with two sided test" was calculated with the help of World Health Organization (WHO) Manual (Lwanga, 1991). The sample size as calculated by sample size calculator was 152 that is, 76 in each group for the validation of result and statistical assessment to be true. Refusal to give consent and dropouts are inherent part of any given clinical trial and in developing world this factor is even more prevalent, so 50 more subjects were added in the calculated sample size to make it 202 (101 in each group).

Sampling procedure

The selected patients were randomized by adopting blocking technique. This technique is used in clinical trial, to ensure that both treatment groups are similar in size (Crawford, 2009). The patients were divided into groups of 10. Marked papers were prepared by a person who was not part of research team. Half (five) of each block of 10 were marked "Treatment Group 1" (TR1) and the rest marked as "Treatment Group 2" (TR2). Each eligible participant was invited to pick blindly, one sheet out of 10 available for example, in one draw, first patient picked TR1, second also picked TR1, third picked TR2, fourth picked TR1, fifth picked TR2, sixth picked TR2, seventh picked TR2, eighth picked TR1, ninth picked TR1 and tenth was automatically allocated to TR2.

Once a sheet was picked up, after noting the treatment group allocation, it was put back in the drawer to make it 10 again so that every patient has an equal chance of being allocated in any of the treatment group. The procedure was repeated for second block of 10 and so on until all patients meeting inclusion/exclusion criteria were registered. These sheets were pulled out by the patient from a drawer at the time of informed consent, so allocation was concealed. Moreover physician and laboratory person were also blinded for the type of treatment. All patients were instructed to return back after following full course of treatment. However they were advised to report back immediately in case there is any side effect or any complication.

Variables studied

Age, sex, education were main demographic variables. Stool was examined to confirm the diagnosis. Other variables included history of chronic disease, presenting complaints, general examination, investigations, treatment option, outcome such as success and failure, side effects etc.

Treatment group 1

This is a combination of metronidazole + diloxanide furoate available in Pakistan with brand name "Entamizole DS". This tablet contains metronidazole 400 mg + diloxanide furoate 500 mg. Entamizole DS was given 3 times a day for 5 days.

Treatment group 2

This involved the use of Endemali®, a herbal product, available in 4 g sachet, containing *Boswellia glabra* 270.9 mg, *Kaolinum ponderosum* 255 mg, *Ocimum pilosum* 580 mg, *Pistacia terbinthus* 116.1 mg, *Plantago ispagula* 812.7 mg, *Vateria indica* 232.2 mg sweetening agent q.s. Endemali® was given 4 times a day for 10 days.

Criteria for assessment of therapeutic evaluation

Success

Success had two components: Parasitological cure and clinical cure. (1) Parasitological cure: No cyst found in the stool five days after stopping treatment. (2) Clinical cure: Absence (partial or complete) of symptoms after stopping the treatment. Treatment success in our case was defined as:

A. Normalization of abdominal and systemic signs or at least improvement in the symptoms.
B. No trophozoites or cyst in stool.

Failure

Treatment was considered failure if there was cyst found in the repeated sample and/or there was no improvement of the symptoms within two weeks.

Inclusion criteria

All the patients reporting to out patient department (OPD) of the two centers between the ages 5 to 60 years, who were proven cases of amoebiasis, were included in the study.

Exclusion criteria

All patients having congenital malformation or any other infection (as found through lab investigation) were excluded from the study. At the beginning of study it was planned that while taking the history and before registration of patient, at the base line, chronic diseases like tuberculosis etc. and co morbid condition like hypertension and diabetes will be excluded and even infection and co morbid condition revealed later on by laboratory investigation will also be excluded to assess the naive cases only. Those having known hypersensitivity to the drugs were also excluded.

Ethical consideration

The protocol was reviewed and approved by ethical committee of Hamdard College of Medicine and Dentistry (Reference No was CMHCMD/001/2007 dated 16th January 2007) and finally permission was granted by Board of Advanced Studies (BASR), Hamdard University in its 25th meeting held on 19th July, 2007. A voluntary fully informed consent was obtained at the time of enrolment of patient. Later on trial was registered at http://www.controlled-trials.com and the registration number allocated was ISRCTN10942146 (Siddiqui, 2012).

Constraints and compromises

It took a long time (From October, 2008 to December, 2009) for the survey to be completed because it was very difficult to find out the patients who fulfilled the inclusion/exclusion criteria. Some of the patients did not give consent. They were treated with routine procedure but their results were not included in the study. The standard test to differentiate between *E. histolytica* and *E. dispar* are enzyme linked immunosorbrnt assay (ELISA) and polymerase chain reaction (PCR) and recommended for the diagnosis of amoebiasis to capture antigen. Due to insufficient funds, the routine microscopic procedure which is being used as basis for the choice of treatment in most part of the developing world was utilized, and as most of the prescriptions are based on stool microscopy, we used the same for general application. Moreover the focus of study was a comparison of two choices of treatment in the available set of facilities; any kind of bias would have affected both groups equally and not affected the primary objective of study as the allocation of the subject was random. However, a study to compare the two drugs based on the diagnosis of amoebiasis through either ELISA or PCR is necessary.

Data collection procedure

The study was executed in three phases, which are as follows:

Phase I

Patients coming for the OPD treatment in two centers, Shifa-Ul-Maluk Hospital, Gadap and Zahida Medical Centre, North Karachi, with the complaint of pain in abdomen, blood in stool or diarrhea were selected to find out the study subjects.

Training of the research team**:** The physicians and laboratory persons were explained to about the purpose and method of study. Principal investigator himself explained each and every variable in the performa and about the importance and method of consent.

Pre-testing: Pre-testing was carried out two weeks before the actual data collection to find out any ambiguity in the performa in another center, located in North Karachi. All the steps needed for the conduction of the study properly were carried out here also. This gave a chance of, not only a thorough evaluation of the performa, but also its ability to assess the patient’s condition.

Physicians examining the patients were supervised individually and any weakness was noted for later rectification. The data was entered in the computer through statistical package for social sciences (SPSS) software version 20 and analyzed to find out any difficulty during entry and analysis of actual result. The result of the pre-testing was more than satisfactory and did not emanate any significant change in the strategy of the study.

The research instrument: The variables described earlier were transformed into performa. Both close type and open type questions were used for variables in the performa depending upon the type of variable.

Phase II

The actual implementation of clinical trial took place in this phase of the study. All patients reporting to OPD of the two centers between 9.00 am and 1.00 pm, who fulfilled the inclusion/exclusion criteria, were included in the study. Any patient reported to the respective health centers for symptoms of amoebiasis was thoroughly investigated for the presence of cyst or vegetative form of *E. histolytica*. All the proven cases were asked for their consent to be included in the studies after signing informed consent.

Phase III

This phase was devoted to data analysis and report writing. SPSS ver. 20 was used for the purpose of data entry. Level of confidence was set at 95% and the level of significance was set at 5%.

Collection of stool samples: Each person with symptoms of amoebiasis was briefed about the appropriate method of collecting stool. They were given a wide, clean container having tight lid, which allowed ready access and visualization of the stool specimen. The specimens were adequately identified by the labels indicating code no., age, sex, etc. The containers were given to the patient the same day; they reported to OPD and were explained to defecate in the container next morning and not to mix with urine or water. It was made sure that the stools were normally passed and no purgatives were used. It was also made certain that the study subjects were not on any therapy for example, antibiotic, anthelminthic, antidiarrheal agent, antacid and hypertonic salts. The specimens were collected by the principal investigator and his team and transferred as soon as possible to the laboratory to avoid loss of trophozoites. The specimens were examined immediately in the laboratory by well-trained, qualified technicians. “Macroscopically stool samples were examined for color, consistency, reaction, presence of mucus, presence of blood, presence of abnormal matter, presence of undigested food and presence of parasites. Microscopically, each specimen was examined as under: (1) One fresh normal saline preparation, (2) One fresh Lugol’s iodine preparation, (3) Zinc sulfate floatation preparation. Formalin in ether sedimentation method was used for examinations of stools containing fatty substances that interfere with zinc sulfate centrifuged floatation method.

Procedure for stool examination: A small quantity of the selected fresh material was placed on a warm slide with a toothpick applicator or platinum wire, thoroughly emulsified in one or two drops of warm physiologic sodium chloride solution and mounted with a cover glass. In fluid and semi fluid stools, the bloody mucous or tiny specks of tissue were selected and in formed feces the material was scraped from surface in several parts of fecal mass. The preparation was made such that it was slightly opaque but thin enough to allow newspaper print to be legible through it. The specimens were examined first by the low power 16 mm objective and then suspicious objects or selected fields were studied with the high power 40 mm objective. Trophozoites and cysts of protozoa and helminthic eggs and larvae appeared in their natural shapes and colors.

Iodine and supravital staining: The treatment of fresh glass mounts with iodine or supravital staining aids in the differentiation of protozoa. The iodine mount which was made on the same slide as the plain mount was used for the identification of eggs and cysts.

Concentration methods for protozoan cysts and helminthic eggs and larvae: Concentration methods fall into two main classes: sedimentation and floatation each with a number of techniques. Sedimentation is less effective than flotation technique. Hence we used flotation technique. This technique is based on the difference of specific gravity of certain chemical solutions. Sugar, sodium chloride or zinc sulphate were employed chiefly. The eggs and cysts float to the surface in the heavier solutions while fecal matter sinks to the bottom gradually. Zinc sulphate is a preferable solution so we used the same. The optimal timings were 5 to 20 min, since the cysts tend to disintegrate after 30 min.

Zinc sulfate centrifugal flotation technique: This valuable method of concentrating cysts and eggs employs a zinc sulphate solution of S.G 1.180, which was made by dissolving 331 g of granular zinc sulphate technical grade in 1000 ml of water and adjusting to exact S.G. using a hydrometer; filtered through glass wool. For formalized feces a solution of higher S.G. 1.200 was used. It is considered to detect about 80% of eggs and cyst in light infections. It destroys trophozoites but does not impair the morphology of cyst for about an hour.

Procedure: A fine suspension was made by comminuting one gram of freshly passed feces in about 10 ml of luke warm tap water. In order to remove the coarse particles, the suspension was strained through one layer of wet cheese cloth in a funnel into a small test tube, 100 by 13 mm. The suspension was centrifuged for 1 min at 2300. The supernatant fluid was poured off, about 2 ml of water was added, the sediment was broken up by shaking or tapping and additional water was added to fill the tube. The washing and centrifuging was repeated until the supernatant was fairly clear. Usually it was necessary to do it three times. The last supernatant fluid was poured off, about 2 ml of zinc sulfate of specific gravity 1.180 was added, the sediment was broken up and sufficient additional zinc sulfate to fill the tube to the rim was added. A cover glass was placed over the top of the tube, which was centrifuged again for one minute at 2300 cycles/minute. The cover glass was removed and mounted on a clean slide in a drop of Lugol’s iodine solution for microscopic examination” (Siddiqui, 2002).

Drugs used in this study

Entamizole DS contains two drugs Metronidazole and Diloxanide furoate in a strength of 400 mg and 500 mg, respectively.

Metronidazole

Mode of action: It is used to destroy amoebae that have invaded

tissue. It kills trophozoites of Entamoeba histolytica in intestine and tissue but does not eradicate cysts from intestines.

Diloxanide furoate

Mode of action: It is a luminal amebicide and acts primarily in bowel lumen because it is poorly absorbed. It is used to eradicate cysts of *E. histolytica* after treatment of invasive disease.

Endemali

Each strip contains the following herbs;

(a) *Boswellia glabra* (270.924 mg). Mode of action: It was found to improve blood supply to the joints and restore integrity of vessels weakened by spasm.
(b) *Kaolinum ponderosum* (255.443 mg). Mode of action: Harmonization of motility and secretion in digestive disorders of the gastrointestinal tract, for example, digestive weakness.
(c) *Ocimum pilosum* (580.552 mg). Mode of action: Antibacterial, antifungal, anti-inflammatory, antipyretics, hepatoprotective, anti-infective
(d) *Pistacia terbinthus* (116.110 mg). Mode of action: Antitussive, nutritive, diuretic, anti-inflammatory, antirheumatic, appetizer, antiulcerative, hepatoprotective.
(e) *Plantago ispagula* (Husk) (812.727 mg). Mode of action: laxative, antiacidic, anti diuretic, demulcent, antidiarrheal, antihyperlipidemic.
(f) *Vateria indica* (232.221 mg). Mode of action: Antitumor, anti-oxidant, antidiarrheal, astringent, antibacterial, anti-inflammatory, sweetening agents and excipients q.s.

RESULTS

This randomized, double blind clinical trial was conducted to compare herbal medicine Endemali and a combination of allopathic preparation, metronidazole and diloxanide furoate (MDF) in order to evaluate the effectiveness of these medicinal preparation for the treatment of amoebiasis. A total of 202 patients fulfilled the inclusion/exclusion criteria. However a total of 78 in allopathic and 75 in herbal group completed their study (Figure 1). Both groups were compared for the basic characteristics and there was no significant difference between the two with respect to age, weight, height, sex, marital status, body mass index (BMI), race, occupation etc. at alpha level 5%, $P < 0.05$. Mean and standard deviation of the ages for Treatment group 1 and Treatment group 2 were 28 ± 9.3 and 29.24 ± 13.9, respectively. The skewness and kurtosis of the data were 0.79 and 0.15, respectively. The standard error of mean was 0.95 and the variable age was normally distributed. Mean height of the population of interest was 152.3 cm with a standard deviation value of 29.0, while mean height for group one and group two was 156 ± 21 and 149 ± 35, respectively. Mean weight of the population of interest was 59.4 kg with a standard deviation value of ± 13.14 while the mean weights for group one and group two were 58 ± 10 and 60 ± 15.1, respectively (Table 1).

With respect to absence of cyst and trophozoites, 60 out of 78, on allopathic medicines, had no cyst after full course of treatment while 55 out of 75 on herbal medicines had no cyst after the full course of treatment. There was no significant difference between the two groups with respect to cure rate (Table 2). Regarding improvement of symptoms, 21 in allopathic group and 23 in herbal group did not show any improvement but there was no significant difference between the two groups (Table 3). However there was a significant difference regarding side effects reported between two options that is, allopathic and herbal with a chi square value of 27.09 and p value = 0.000 (Table 4).

Forty four (28.8%) patients reported side effects of drugs. Sixteen (10.5%) reported anorexia after medication, 9 (5.9%) metallic taste and 6 (3.9%) headache after medication. Patients receiving allopathic form of treatment reported more side effects than herbal (Table 5).

DISCUSSION

Amoebiasis is one of the commonest problems especially in the developing countries (WHO, 1969). The prevalence rate varies from country to country with as low as 0.2% in endemic areas of developed countries to as high as 40% in developing countries (Haque, 2006; WHO, 1981). It is estimated that 80% of the world population utilize plant as their main source of medicinal agent. Moreover traditional medicine is still the only health source available to most of the world population. The importance of herbal medicine can be realized by the fact that South Africa flora consists of 30,000 species of higher plants and nearly 10% (3000) of these are used in medicine (Taylor, 2001). It is claimed that herbal formulas produce high cure rates with few or no side effects because of the more close formulation to human interior milieu. Moreover it improves the general well-being of the patient and has better compliance (Shahab-ud-din, 2006). Bland (1986) reported that the herbal medicine, though lack defined doses, but have advantage of lesser side effects. However contrary to the claim, as in preceding lines, Ernst (1998) described that herbal products can lead to hypersensitivity reactions ranging from transient dermatitis to anaphylactic shock (Ernst, 1998).

Traditionally, metronidazole is prescribed for the treatment of amoebiasis and taken as drug of choice (Tasanor, 2007; Gupta, 2004; Khairnar, 2007) but studies have shown that it is not effective against the cyst, as it is rapidly absorbed from the lumen (Powell, 1969). The present recommendations are to combine this drug with some luminal amoebicidal like paromycin or diloxanide

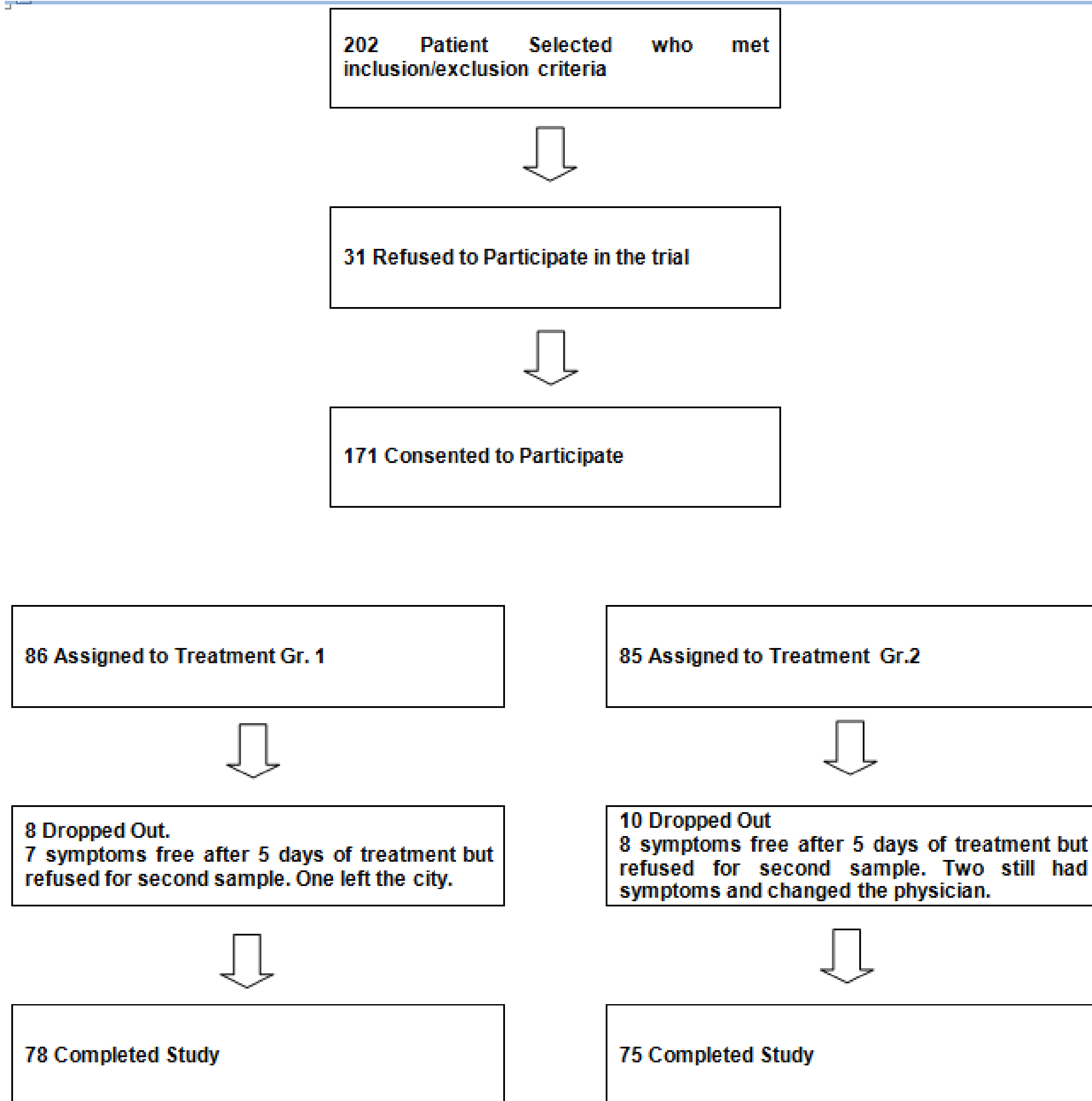

Figure 1. Flow chart demonstrating random allocation of the subjects to two arms of allopathic and herbal treatment.

furoate. Though metronidazole is effective in treating the amoebiasis but it resulted in failure due to development of resistance by *E. histolytica* against it (Orozco, 2002; Gonzales, 2009).

This study used combination of metronidazole and diloxanide furoate (MDF) as the drug of choice from allopathic form of treatment and Endemali, a herbal product, to compare the cure rate and side effects of the two products.

In the present study, we enrolled 202 patient confirmed for the diagnosis of amoebiasis but only 153 completed their study which included 78 on allopathic and 75 on herbal therapy. As the drop out was considered as potential factor in the design phase an extra sample of 50 patients proved sufficient to meet the required number for the application of test of statistical significance. Both

Table 1. Distribution of base line Characteristics of the two treatment groups.

Characteristics	Treatment Group 1 Allopathic (n=78)	Treatment Group 2 Herbal (n=75)	Test of significance	P value
Age	28±9.3	29.24±13.9	0.357 Student t test	0.759
Weight	58±10.	60±15.1	1.041 Student t test	0.300
Height	156±21	149±35	0.361 Student t test	0.719
Race				
Sindhi	15	12	7.3 (Fischer Exact)	0.126
Punjabi	15	15		
Urdu	27	33		
Pashto	9	3		
Balochi	9	12		
Any other	3	0		
Sex				
Male	42	48	1.632 (Chi Square)	0.201
Female	36	27		
Body Mass index (kg/m^2)	23.07+3.29	23.84+3.09	2.245 (ANOVA)F	0.136
Marital status				
Married	27	36	2.8 (Chi Square)	0.10
Unmarried	51	39		
Occupation				
Student	21	30	5.62 (Chi Square)	0.22
House wife	15	9		
Sales man	9	9		
Gardner	3	6		
Any other	30	21		
History of Move				
Yes	15	9	1.51 (Chi Square)	0.219
No	63	66		

groups were compared for the homogeneity by applying test of significance, at baseline and no significant difference was found between the two groups of treatment with respect to confounding variables, like, socio-economic and demographic variables. We used mean, standard deviation, Student t-test, Chi square test, Mann-Whitney U test, Kendall tau-b and analysis of variance (ANOVA) for comparison of data, depending upon the nature of grouping variables.

Our result for mean age of the population of interest, 28.94, are less than Mohiuddin (2007) (33 years) and in agreement with Shahab (2006) (28 years). It was probably due to the reason that we included patients from age 5 to 60 years while Mohiuddin (2007) included from a minimum of 15 to 68 years. However all three studies result demonstrates that disease affects young and productive age group in the population, resulting in economic losses. Regarding the cure rate we found no significant difference between MDF and Endemali while Shahab (2006) reported a significantly better result with MDF as compare to Endemali. About 25% patients were passing cyst even after completing the full course of therapy in both the groups with no significant difference between the two treatment groups.

Table 2. Treatment option by presence of cyst after treatment.

Characteristics	Category	Cyst present	Cyst absent	Chi square value	P value
Treatment option	Allopathic	18	60	0.26	0.6
	Herbal	20	55		

Table 3. Treatment option by type of improvement.

Characteristics	Category	Allopathic	Herbal	Kendall's tau-b	P value
Slight improvement	Slight improvement	14	6	0.004	0.959
	Moderate improvement	19	22		
	Completely recovered	24	24		
	No improvement	21	23		

Table 4. Distribution of side effects by treatment options.

Side effects reported	Treatment option		Total
	Allopathic	Herbal	
Yes	37	7	44
No	41	68	109
Total	78	75	153

Pearson Chi-Square Value = 27.094 p value < 0.000.

Table 5. Distribution of types of side effects by treatment option.

Types of side effects	Treatment option		Total
	Allopathic	Herbal	
Anorexia	14	2	16
Metallic taste	7	2	9
Headache	5	1	6
Flatulence	5	0	5
Pain in abdomen	4	1	5
Any other	2	1	3
Total	37	7	44

Tasanor (2007) tested *in vitro* three of the allopathic drugs as control (Metronidazole, Dehydroemetine and Dihydroartiminisin) and seven herbs (leaves, root and stem bark of *Agalia eleagnoidae* (Ae), leaves from *Stemona tuberose* (St), stem bark and leaves of *Agalia odutis* (Ao), and leaves of *Agalia odorata* (Aod). Stem bark extract of Ae demonstrated the highest activity with the lowest IC99 (495.5 ng/ml) and the steepest slope 1.1325 (Tasanor, 2007). The study showed a better response of all herbal medicines as compared to Metronidazole, tested against *E. histolytica*.

There was a highly significant difference between the side effects reported by allopathic form of treatment than its herbal counterpart in the current study. However the symptoms in no case were serious enough to drop out of the study. The most common side effect reported was anorexia (n = 14) followed by metallic taste (n = 7) in the allopathic group while herbal medicine presented with the same side effects that is, anorexia (n = 2) and metallic taste (n = 2).

The result of the study depicts that null hypothesis for the effectiveness of two drugs, cannot be rejected. However the null hypothesis for the side effects of the two drugs was rejected with a highly significant difference and Endemali had significantly less side effects as compared to MDF.

Conclusion

Based on this study, it is concluded that, there is no significance difference between the cure rate of MDF and Endemali. So both drugs are equally effective in treating amoebiasis. Endemali is a better tolerated drug and friendlier to internal environment of human body as there were significantly more side effects reported due to MDF as compared to Endemali. Both the drugs had high failure rate, hence there is a need to search for new salts for this killer disease. This study may be taken as a platform for the development of new therapeutics as we have used gold standard for the clinical trial protocol that is, double blind clinical trial.

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Conflict of interest

Authors declare that there are no conflicts of interest.

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Full Length Research Paper

Phytochemical, antioxidant and cytotoxic analysis of Brazilian cerrado plants: Preliminary evidence of their antitumor activity

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The aim of this study was to analyze the antitumor activity of *Anacardium humile* A. St.-Hil., *Brosimum gaudichaudii* Trécul and *Tabebuia avellanedae* Lorentz ex Griseb species, which are distributed in the Cerrado region of Brazil. Qualitative and quantitative analyses were also conducted on the bark and leaf extracts and antioxidant and cytotoxic activities were studied. The results showed that the bark and leaf extracts of *A. humile* exhibited the highest contents of phenolic compounds and optimal antioxidant activity. The leaf extract of *T. avellanedae* had the highest content of flavonoids, displayed high efficiency as a ferrous ion chelating agent and inhibited the growth of the human laryngeal carcinoma (HEp-2) cell line by 88.0% at the concentration of 50 µg/ml. The leaves of *B. gaudichaudii* had higher levels of condensed tannins, flavonoids and coumarins compared with that of the bark. There was a correlation between inhibition of the NCI- human lung cancer (H292) cell line and the content of condensed tannins and antiradical activity and also between the inhibition of human colon carcinoma (HT-29) cell growth and the antioxidant and chelating activity of ferrous ion. Our data provide evidence that bark and leaf extracts of *A. humile* and leaves of *T. avellanedae* possess anticancer activity; therefore, the isolation and identification of the active ingredients of these plant tissues should be conducted to explore their therapeutic potential.

Key words: Antiproliferative, Hep-2 cells, HT-29 cells, medicinal plants, NCI-H292 cells, phenolic compounds.

INTRODUCTION

The search for more effective and less toxic cancer drugs remains a challenge, and despite the development of organic synthesis, approximately 75% of prescribed medications are derived from plants (Tan et al., 2006),

demonstrating that plants are a source of new drugs for currently incurable diseases, such as cancer (Mahata et al., 2012). Medicinal plants are important natural sources of medicines, and they have the potential to provide prototype molecules related to the diversity of their constituents (Yunes and Calixto, 2001).

Although numerous studies have evaluated the biodiversity richness of Brazilian flora, little is known of the pharmacological potential of the plants that occur in the different ecosystems (Albuquerque et al., 2007; Di Stasi et al., 2002), especially plants belonging to the Cerrado biome. The species *Anacardium humile* is a perennial plant that is widely distributed throughout the Brazilian Cerrado regions. Although the leaves of *A. humile* are used to treat general inflammation and diarrhea, limited studies have evaluated the phytochemical and biological activities of this plant. Another species native to and widely dispersed in the Brazilian Cerrado biome is *Brosimum gaudichaudii* Tréc, which belongs to the family Moraceae. According to Conceição (1980), this plant is popularly used for the treatment of skin blemishes and as a detoxicant and blood purifier (Barros, 1997). The main therapeutic use of *B. gaudichaudii* is in the treatment of vitiligo (marketed as Viticromin®); however, limited data are available on its experimental toxicity apart from its demonstrated clinical efficacy (Cunha et al., 2008) and antitumor activity. Plants belonging to the genus *Tabebuia* have wide distribution in the America, and in the bark and core of the stem, variable amounts of lapachol and other similar substances are found that are endowed with strong bactericidal (Park et al., 2005), fungicidal (Portillo et al., 2001), antiophidic (Núñez et al., 2004) and antitumor (Di Chenna et al., 2001) activity. However, studies on *Tabebuia avellanedae* found in the Brazilian Cerrado are still scarce.

Studies on medicinal plants have been reoriented recently because of the emergence of high technology analytical methods that can isolate plant components even at low quantities and determine the mechanisms of action of the isolated plant extracts and compounds (Yunes and Cechinel Filho, 2007). Cytotoxicity and antitumor activity analyses that demonstrate the pharmacological potential of medicinal plants are among these new methods, and both are related to the potential anticancer activity of the plant extracts. Similarly, insights into potential mechanisms related to the antitumor action of extracts are possible through phytochemical analyses that can quantify phenolic compounds (such as tannins and flavonoids) and determine their antioxidant capacity, especially in the sequestration of free radicals (Govindarajan et al., 2005; Pereira et al., 2009).

Therefore, because the Cerrado has one of the greatest biodiversity of plants among the different Brazilian biomes (Guarim Neto and Morais, 2003) and there are a limited number of studies on the therapeutic potential of these plants, especially on potential antitumor activities, the aim of this study was to analyze the phenolic content, antioxidant capacity and cytotoxic activity of the ethanol extracts of the leaves and barks of three plant species distributed in the Brazilian Cerrado to determine their potential for pharmacological action important for growth inhibition of tumor cell lines.

MATERIALS AND METHODS

Sample collection and preparation of extracts

Plant material for the preparation of voucher specimens and production of the extracts was collected from April 9 to 11, 2012 in the city of Palmas, Tocantins State, Brazil (10°10'58.45" S and 48°17'33.75" W). The bark and leaves of the following species were collected: *A. humile*, locally known as cajuzinho; *B. gaudichaudii*, popularly known as inharé; and *T. avellanedae,* popularly known as purple ipê. These species were chosen because they occur in high numbers throughout the Cerrado and there is a lack of studies analyzing their medicinal potential.

The plant parts were dried in a forced air oven at 40 ± 2°C, pulverized in a vertical knife mill and then standardized in sieves (#18 US mesh) to yield a grain size of 1 mm. The extracts were prepared by maceration with ethanol for 48 h (1:20, w/v), and then the material was filtered through filter paper and evaporated under reduced pressure at 40 ± 2°C until a dry extract was formed (Araújo et al., 2012).

Phytochemical profile

The qualitative analysis of the extracts (10 mg/ml) was performed by silica gel thin-layer chromatography using specific eluent and revealing systems according to Wagner and Bladt (1995) (Table 1). The presence of saponins was confirmed by the formation of persistent foam for 10 min then 5 mg of extract was stirred with 10 ml water.

Content of phenolic compounds

The total phenolic content was determined in test tubes with 0.5 ml of the diluted extracts (500 µg/ml), 0.5 ml of 10% (v/v) Folin-Ciocalteu reagent (Fluka) aqueous solution and 1 ml of 7.5% (w/v) sodium carbonate (Fmaia) aqueous solution, and then distilled water was added to a final volume of 10 ml. The samples remained at rest for 30 min and the absorbance was read at 760 nm. The assays were performed with six replicates. The total phenolic content was expressed in mg tannic acid (Vetec) equivalent per gram (TAE/g) extract (Peixoto Sobrinho et al., 2008).

The condensed tannin content was quantified according to Burns (1971) and adapted for the species. One milliliter of the diluted extract (500 µg/ml), 4 ml of methanol, 2.5 ml of 10% (v/v) hydrochloric acid (Fmaia) solution in methanol, and 2.5 ml of 1% (w/v) vanillin-methanol (Vetec) solution were transferred to test tubes. The mixture was heated at 60°C for 10 min, and then the absorbance was read at 730 nm. The content of condensed tannins

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Table 1. Phytochemical prospecting according to Wagner and Bladt (1995).

Group	Solvent systems	Standard	Chromogenic agents	Detection (λ)
Alkaloids	Toluene:Ethyl acetate:Diethylamine (70:20:10)	Cinconin and yohimbine	Dragendorff	Brown or orange-brown (Vis)
Phenolic compounds	Ethyl acetate:Glacial acetic acid:Formic acid:Water (100:11:11:26)	Rutin and quercetin	NEU	Flavonoids: orange or yellow (UV-365) Cinnamic acid derivatives: deep blue (UV-365) Phenylpropanol glycosides: lime green Condensed tannins: blue (UV-365)
Coumarins	Toluene:Petroleum ether (1:1, acetic acid 10%)	1,2-benzopyrone	KOH ethanolic 10%	Blue and blue-green (simple coumarins); yellow, blue and brown (derivatives) (UV-365)
Anthracene derivatives	Ethyl acetate:Methanol:Water (100:13.5:10)	Aloin	KOH ethanolic 10%	Anthraquinones: red (Vis/UV-365) Anthrone and anthranol: yellow (Vis/UV-365) Aloe resin: blue (Vis/UV-254/UV-365)
Monoterpenes, sesquiterpenes and diterpenes	Toluene:Ethyl acetate (97:3)	Thymol and carvacrol	Vanillin sulfuric10 min, 110ºC	Bue, violet, orange, green, red and brown (Vis)
Naphthoquinones	Toluene:Formic acid (99:1)	Lapachol	KOH ethanolic 10%	Violet to brown (Vis) Yellow to brown (UV-365)
Triterpenes and steroids	Toluene:Chloroform:Ethanol (40:40:10)	Lupeol (triterpenes) and sitosterol (steroids)	Lieberman-Burchard 5-10 min, 110ºC	Gray to red-brown (Vis) Blue, blue-violet (UV-365)
Xanthines	Ethyl acetate:Methanol:Water (100:13.5:10)	Aloin, caffeine and theobromine	Iodine - KI - HCl	Brown (Vis)

was expressed as mg catechin (Sigma-Aldrich) equivalent per gram (CE/g) extract (Burns, 1971). The content of total flavonoids was determined from the mixture of 0.5 ml of diluted extract (500 µg/ml), 0.5 ml of 60% (v/v) acetic acid-methanol solution (Merk), 2 ml of 20% (v/v) pyridine-methanol solution (Vetec), 1 ml of 5% (w/v) aluminum chloride in methanol (Honeywell), and 6 ml of distilled water in test tubes. The samples remained at rest for 30 min, and the absorbance was read at 420 nm. The content total flavonoids was expressed as mg rutin (Acros) equivalent per gram (RE/g) extract (Peixoto Sobrinho et al., 2008).

The content of coumarins was evaluated according to the method described by Osório and Martins (2004). Briefly, 0.5 ml of the diluted extract (500 µg/ml) was transferred to test tubes and 2 ml of distilled water and 0.5 ml of a 5% (w/v) lead acetate aqueous solution were added. The sample was stirred and 7 ml of distilled water were added. Two milliliters of this solution were transferred to a test tube and 8 ml of hydrochloric acid solution (Fmaia) (0.1 M) was added. Samples remained at rest for 30 min and the absorbance was read at 320 nm. The content of total coumarins was expressed as mg coumarin (Sigma-Aldrich) equivalent per gram (CoE/g) extract.

Quantification of the antioxidant activity by the DPPH method

The quantification of antioxidant activity was performed using the 1,1-diphenyl-2-picrylhydrazil (DPPH) (Sigma-Aldrich) assay according to the protocol adapted from Sousa et al. (2007). Three independent samples were

prepared at a concentration of 500 µg/ml in methanol (Vetec). From these samples, six dilutions were prepared to yield concentrations ranging from 10 to 250 µg/ml depending on the antioxidant capacity of the extract. When the species exhibited higher antioxidant potential, smaller concentrations were used to obtain an exponential curve because higher concentrations would saturate the DPPH solution (Merk), thereby producing similar absorbance values.

Subsequently, a solution of DPPH (40 µg/ml) in methanol was prepared. From each concentration, 0.5 ml of plant extract was removed and mixed with 3.0 ml of the DPPH solution in a test tube. After 30 min, the absorbance of this solution was read at 517 nm in duplicate for each concentration of the three independent samples. Ascorbic acid (Nuclear) was used as a positive standard at concentrations between 5 and 50 µg/ml following the same procedure described for the extracts.

Using the different concentrations, the effective concentration (EC_{50}), which is the concentration required to inhibit or decrease the initial concentration of DPPH by 50%, was calculated. To calculate the EC_{50}, concentrations of the positive standard or samples are used as the independent variable (µg/ml) and the percentage of remaining DPPH (% $DPPH_{REM}$) is used as the dependent variable to produce a first-order exponential curve and an equation to calculate the effective concentration (Sousa et al., 2007):

$$DPPH_{REM} (\%) = (DPPH]_{T=t} / DPPH]_{T=0}) \times 100$$

where $DPPH]_{T=t}$ is the concentration of DPPH after the reaction with the extract and $DPPH]_{T=0}$ is the initial concentration of DPPH, that is, 40 µg/ml (100 µmol/ml) (Sousa et al., 2007).

Analysis of the antioxidant activity: ferrous ion chelating activity

The method to analyze the antioxidant – ferrous ion chelating (FIC) activity was adapted from Chew et al. (2009) and Santos et al. (2007). Transferred to test tubes were 1 ml of the diluted extract at concentrations between 250 and 5000 µg/ml and 1 ml 0.1 mM (w/v) ferrous sulfate solution (Vetec) in methanol, and after 10 min, 1 ml 0.25 mM (w/v) ferrozine solution (Sigma-Aldrich) in methanol was added, and the samples remained at rest for 10 min. The absorbance was read at 562 nm using 1 ml of each concentration and 2 ml 75% methanol as a blank.

For this test, ethylenediaminetetraacetic acid (EDTA) (Fmaia) was used as a positive standard at concentrations between 5 and 50 µg/ml. The analyses were performed in triplicate, and the positive standard was prepared with 1 ml of each reagent (75% (v/v) methanol, ferrous sulfate and ferrozine) following the same order of addition and time interval.

For each concentration, the percentage of the sample capable of FIC was calculated according to the following equation:

$$\text{Chelating activity} (\%) = A_{control} - (A - Ab) / A_{control} \times 100$$

where $A_{control}$ is the mean absorbance of the control; A is the absorbance of the test sample; and Ab is the absorbance of the blank at the same concentration as the test sample.

A logarithmic curve was produced by plotting the concentration versus the percentage of chelating activity, and EC_{50} was determined from the resulting equation.

Cytotoxic evaluation

The cytotoxic activity was evaluated using the colorimetric method. These assay is based on the capacity of mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells (Alley et al., 1988; Mosmann, 1983).

The cell lines human colon carcinom (a HT-29), human laryngeal carcinoma (HEp-2) and human lung cancer (NCI-H292), obtained from the Cell Bank of Rio de Janeiro, were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco); the cell lines acute promyelocytic leukemia (HL-60) and human breast adenocarcinoma (MCF-7) were maintained in RPMI-1640 medium (Himedia); and the cell line human mammary gland adenocarcinoma (SK-BR-3) was maintained in McCoy's 5A medium (Sigma – Aldrich). All media were supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% Pen Strep (Gibco) and maintained at 37°C and 5% CO_2.

An initial screening was conducted to determine the cytotoxic potential of the extracts at a concentration of 50 µg/ml. The cells HEp-2, NCI-H292, HT-29, MCF-7, SK-BR-3 (10^5 cells/ml) and HL-60 (0.3×10^6 cells/ml) were plated in 96-well plates and incubated for 24 h. Subsequently, the extracts were dissolved in 1% dimethyl sulfoxide (DMSO) (Himedia) and culture medium and added to the wells, and the plates were incubated for an additional 72 h. Doxorubicin (5 µg/ml) (Eurofarma) was used as positive control. Then, 25 µl of MTT (5 mg/ml in phosphate buffered saline - PBS) (Himedia) were added to the wells, and after 3 h of incubation, the culture medium with MTT solution was aspirated and 100 µl of DMSO was added to each well. The absorbance was read on an automatic microplate reader at 560 nm after dissolution of the formazan crystals (Alley et al., 1988). The mean optical density (OD) of the samples was compared with the mean OD of the control (DMSO 1%).

These experiments were performed in triplicate, and the percentage of inhibition was calculated using the program GraphPad Prism 5.0. The percentage growth inhibition was calculated using following formula:

$$\text{Cell inhibition} (\%) = 100 - \{(At - Ab)/(Ac - Ab)\} \times 100$$

where At = Absorbance value of test compound; Ab = Absorbance value of blank, Ac = Absorbance value of control.

Statistical analysis

From the phytochemical profile, the similarity between the bark and leaf extracts was analyzed; the extracts were considered similar when the same compound was present (+) or absent (-) in both the bark and leaf. This similarity was expressed as a percentage based on the 14 groups of secondary metabolites that were qualitatively tested.

The total phenol, condensed tannin, total flavonoid and coumarins content and antioxidant activity were correlated using Spearman's correlation to test for correlations among the phenolic compounds, between the compounds and antioxidant activity, and among the types of antioxidant activity in the set of analyzed species. Correlation tests with the values obtained from the cytotoxic evaluation of the six cell lines were also performed for these parameters to test if the cytotoxic activity was associated with these parameters.

An analysis of variance (ANOVA) followed by Tukey's multiple comparison test were performed to check for differences between the tested extracts based on the four quantitative analyses of the phenols and two antioxidant activity assays. This statistical analysis was designed to quantitatively identify the most promising extract or level of antioxidant efficiency when significant differences were observed among the values of these parameters for each extract. For all of the statistical tests, $p<0.05$ was adopted and the software

Table 2. Phytochemical prospecting of the hydroethanolic extracts from Cerrado species.

Compound	*Anacardium humile*		*Brosimum gaudichaudii*		*Tabebuia avellanedae*	
	Bark	Leaves	Bark	Leaves	Bark	Leaves
Alkaloids	-	+	+	+	-	+
Aloe resin	-	-	-	-	-	-
Anthraquinones	-	-	-	+	-	+
Anthrone and anthranol	+	-	+	-	-	-
Coumarins	+	+	+	+	+	+
Cinnamic acid derivatives	+	-	-	-	-	-
Phenylpropanol glycosides	-	+	+	+	+	-
Flavonoids	+	+	+	+	+	+
Monoterpenes, sesquiterpenes and diterpenes	-	+	+	+	+	+
Naphthoquinones	-	-	+	+	+	+
Saponins	+	+	+	-	-	+
Condensed tannins	+	+	+	+	+	+
Triterpenes and steroids	-	-	+	-	+	+
Xanthine	+	+	+	+	-	-

(+)Presence of the compound; (-) absence of the compound.

BioEstat 5.0 was used (Ayres et al., 2007).

RESULTS

Phytochemical profile

The thin-layer chromatography analysis (Table 2) revealed that the extracts from the bark and leaves of *B. gaudichaudii* and *T. avellanedae* had greater phytochemical similarity because 10 classes of compounds (71.43%) of the 14 analyzed were absent or present simultaneously in these parts. The extracts of *A. humile* exhibited 64.29% phytochemical similarity between the bark and leaves.

The extract from the bark of *B. gaudichaudii* displayed the greatest diversity of secondary metabolites with the presence of 11 classes, and it was followed by leaf extracts both from *B. gaudichaudii* and from *T. avellanedae*, with 9 classes. Coumarins, flavonoids and condensed tannins were found in all of the extracts. There was a high incidence of monoterpenes, sesquiterpenes and diterpenes, although these compounds were not detected in the bark extract from *A. humile*. Compounds with the lowest frequency were cinnamic acid derivatives, which were only observed in *A. humile* bark extract. No extract showed the presence of aloe resin.

Phytochemical analyses

Quantitative phytochemical analysis showed that *A. humile* had the highest ($p<0.05$) levels of total phenolic content and condensed tannins in the extracts (Table 3) and *B. gaudichaudii* had the second highest total phenolic content (380.22 ± 42.53 and 302.84 ± 19.06 mg TAE/g) and condensed tannins (229.33 ± 9.32 and 309.67 ± 23.73 mg CE/g) in the bark and leaf, respectively.

In the three species analyzed, the total phenolic content was higher in the bark than in the leaves. However, although the content in the bark of *A. humile* was quantitatively higher (566.11 ± 25.41 mg TAE/g) than in the leaves (525.09 ± 22.58 TAE/g), the difference between the parts was not significant ($p>0.05$). The leaf extracts from *B. gaudichaudii* and *T. avellanedae* exhibitted higher levels of condensed tannins compared to that of the bark ($p<0.05$). All of the leaf extracts exhibited a higher content of flavonoids than did the bark extracts, and the leaf extract of *T. avellanedae* stood out because it exhibited a mean 185.71 ± 12.40 mg RE. In all of the species analyzed, there was little variation in the content of coumarins; thus, no significant differences were observed ($p>0.05$).

Antioxidant analysis

Regarding the antioxidant activity, the extracts of *A. humile* showed the highest efficiency in the sequestration of free radicals with a mean EC_{50} of 19.34 ± 0.99 µg/ml for the bark and 23.58 ± 0.52 µg/ml for the leaves (Table 3). In general, the extracts that had the highest total phenolic content and, more specifically, tannin content, such as the extracts of *A. humile*, showed better results

Table 3. Quantification of total phenols, condensed tannins, total flavonoids, coumarins and antioxidant activity of species collected in an area of the Brazilian Cerrado.

Scientific name	Part	Phenolic compound				Antioxidant activity	
		Content of total phenols (mg TAE/g)	Content of condensed tannins (mg CE/g)	Content of flavonoids (mg RE/g)	Content of coumarins (mg CoE/g)	DPPH (EC_{50} µg/ml)	FIC (EC_{50} mg/ml)
Anacardium humile	Bark	566.11 ± 25.41[a]	423.67 ± 7.94[a]	23.43 ± 2.60[a]	70.83 ± 8.32[a]	19.34 ± 0.99[a]	3.57 ± 0.63[a]
	Leaves	525.09 ± 22.58[a]	348.33 ± 12.69[b]	71.43 ± 4.16[b]	79.16 ± 1.90[a]	23.58 ± 0.52[a]	898.90 ± 469.53[b]
Brosimum gaudichaudii	Bark	380.22 ± 42.53[b]	229.33 ± 9.32[c]	59.43 ± 3.73[b]	62.08 ± 16.12[a]	27.17 ± 0.38[a]	247.92 ± 67.78[c]
	Leaves	302.84 ± 19.06[c]	309.67 ± 23.73[d]	138.29 ± 4.45[c]	80.41 ± 7.10[a]	26.50 ± 2.95[a]	6.52 ± 1.30[a]
Tabebuia avellanedae	Bark	128.29 ± 3.73[d]	109.33 ± 4.94[e]	32.00 ± 1.63[a]	78.75 ± 11.45[a]	128.54 ± 2.12[b]	-
	Leaves	55.27 ± 20.53[e]	179.33 ± 5.08[f]	185.71 ± 12.40[d]	57.50 ± 2.16[a]	355.05 ± 32.31[c]	19.85 ± 15.12[a]
Positive standard	-	-	-	-	-	18.04 ± 2.09[a]	14.78 ± 0.23[a]

Mean values followed by standard deviation; mean ± standard deviation. The same letters in the same column indicate no significant difference by one-way ANOVA (Tukey's test) at p<0.05. Test not performed (extract was not soluble).

in the sequestration of free radicals. The statistical analysis revealed an inverse correlation between the content of total phenols and condensed tannins in the sequestration of free radicals (rs = -0.9429 and -0.9429, respectively; $p<0.05$); thus, when these compounds are found in high amounts, a lower concentration is needed to sequester free radicals.

Moreover, the comparative analysis of the parts of each plant showed that only *T. avellanedae* exhibited a significant difference in the sequestration of free radicals ($p<0.05$) and the bark extract (128.54 ± 2.12 µg/ml) was more efficient than the leaf extract (355.05 ± 32.31 µg/ml). For *A. humile* and *B. gaudichaudii*, both the bark and leaves were equally efficient in sequestering free radicals, and no significant difference ($p>0.05$) was observed between the extracts for ascorbic acid, which was used as a positive standard.

The bark extract of *A. humile* and leaf extract of *B. gaudichaudii* and *T. avellanedae* stood out with respect to FIC activity, with the latter two statistically similar ($p<0.05$); however, the bark extract of *A. humile* exhibited lower EC_{50} values (3.57 ± 0.63 mg/ml). The three extracts did not differ with respect to EDTA, which was used as a positive standard (Table 3). In contrast to the observed antioxidant activity in the sequestration of free radicals, there was no correlation between FIC activity and phenolic compounds. The bark extract of *T. avellanedae* was not soluble in the solvent that was used; therefore, it was excluded from the analysis.

Cytotoxic evaluation

Analyses to determine the inhibition of cell lines exposed to the extracts demonstrated that higher percentage of inhibition was observed for the leaf extract of *T. avellanedae*, which inhibited the growth of the HEp-2 cell line by 88% and HL-60 cell line by 67.54%. The extracts from the bark and leaves of *A. humile* and bark of *B. gaudichaudii* inhibited the growth of the MCF-7 cell line by 60.64, 65.98 and 66.29%, respectively (Table 4).

From the correlation analysis of the quantified phenol parameters and two antioxidant activity tests for cell inhibition, it was observed that the values for condensed tannins and antioxidant activity in the sequestration of free radicals (DPPH) were correlated with the inhibition of the NCI-H292 cell line (rs = 0.871 and -0.871, respectively; $p<0.05$) and FIC antioxidant activity was correlated with the inhibition of the HT-29 cell line (rs = -0.828; $p<0.05$). These results suggest that the inhibited growth of these cell lines may be explained in part by the content of condensed tannins, sequestration of free radicals (DPPH) antioxidant activity, and FIC antioxidant activity.

DISCUSSION

Phytochemical and antioxidant analyses

The species of the family Anacardiaceae are known for having high concentration of phenolic

Table 4. Percent growth inhibition of cells subjected to extracts (50 µg/ml) from species collected in the Brazilian Cerrado.

Sample	Part	Cell growth inhibition (%)					
		NCI-H292	HL-60	MCF-7	HT-29	HEp-2	SKBR-3
Anacardium humile	Bark	34.55	20.76	60.64	37.43	9.60	43.70
	Leaves	32.01	13.53	65.98	4.59	14.40	33.90
Brosimum gaudichaudii	Bark	29.08	33.27	66.29	10.59	33.90	45.10
	Leaves	34.47	3.70	34.53	10.27	26.50	48.40
Tabebuia avellanedae	Bark	10.59	27.01	19.42	11.93	3.10	23.80
	Leaves	27.30	67.54	41.93	55.69	88.00	39.50
Doxorubicin	-	94.15	92.91	74.77	64.12	79.39	75.03

compounds and, in particular, tannin compounds (Araújo et al., 2008; Chaves et al., 2010; Boni et al., 2014), which is consistent with our study, which demonstrated that the bark and leaves of *A. humile* exhibited higher contents of phenolic compounds and tannins when compared with that of the other studied species. According to the phytochemical profile, many of the compounds present in the bark and leaf extracts of other species of this taxon (*Anacardium occidentale* L.) were characterized, including saponin glycosides, flavonoids, alkaloids, tannins and phenolic compounds, anacardic acids (monoene and diene), beta-sitosterol, and stigmasterol (Chaves et al., 2010; Nery et al., 2010; Jaiswal et al., 2012). However, limited studies have assessed the phytochemical profile of *A. humile*, especially for the bark of this species. Regarding its leaves, Luiz-Ferreira et al. (2010) demonstrated that the ethanolic leaf extract contained phenolic compounds, gallic acid derivatives, catechins and flavonoids (flavonoid glycosides and biflavonoids), whereas the ethyl acetate fraction contained a high concentration of tannin compounds (Ferreira et al., 2012).

In our study, a correlation was observed between the presence of phenolic compounds and sequestration of free radicals and the inhibition of NCI-H292 cell line growth as well as an association between the FIC activity and the inhibition of HT-29 cell line growth. These associations suggest that both the leaf and bark extract of *A. humile* can be good candidates for the isolation of antitumor active compounds because they exhibited a high content of phenolic compounds and high antioxidant activity.

It has been demonstrated that high content of condensed tannins in grape seed extracts exerts anticancer effects inhibiting the growth of the human colon cancer cell line Caco-2 (Di Nicola et al., 2010). Moreover, apoptosis was enhanced through both caspase-dependent and caspase-independent mechanisms, leading to an early apoptosis-inducing factor release and, further, to a dramatic increase in caspase 7 and 3 activity on Caco2 and HCT-8 colon cancer cells (Di Nicola et al., 2012).

Pereira et al. (2009) reported that tannins, such as metal chelators and those involved in the sequestration of free radicals, act as antioxidants because of their ability to bind to and suppress the enzymatic activity of proteins. The latter effect is increased in compounds with a larger number of galloyl groups and higher molecular weight and those that display an ortho-dihydroxy structure because these characteristics are responsible for the chelating activity and ability to sequester radicals, which may explain, in part, the antioxidant action observed in the bark and leaves of *A. humile*.

Furthermore, the bark and leaves of *A. humile* exhibited phytochemical similarity, indicating that both plant parts of this species have similar therapeutic potential. In our study, the analyses of *A. humile* were performed with crude extracts, and a subsequent fractionation of these extracts may increase the efficiency of growth inhibition of the cancer cell lines by increasing the concentration of active compounds for this type of biological assay. Therefore, our data suggest that *A. humile* is a promising species in the search for new cancer drugs.

Studies with *B. gaudichaudii* have reported the presence of phenolic compounds in all of the plant structures, including the reproductive parts, and the production of these compounds can be demonstrated by the presence of phenolic idioblasts (Jacomassi et al., 2007, 2010). Among these compounds, the best known are bergapten and psoralen, which are two furocoumarins used in the treatment of vitiligo that can be found in the bark and stem (Neves et al., 2002). Our study demonstrated that the leaves of *B. gaudichaudii* have the potential for use as an alternative when searching for therapeutically active compounds because they exhibited higher mean values of condensed tannins, flavonoids

and coumarins than the bark.

The bark and leaf extracts of *B. gaudichaudii* showed no significant results for the inhibition of tumor cell growth, except inhibition rate (66.29% on the MCF-7 cell line) found in the bark extract. Few studies have assessed the potential antitumor activity of the compounds in the bark and leaves of *B. gaudichaudii*. However, the roots were reported to contain cinnamic acid, propanoic acid, coumarins, chalcone, steroids and triterpene (β-amyrin) (Monteiro et al., 2002), and these extracts show low acute toxicity in *in vivo* studies (Cunha et al., 2008). Of the previously identified root compounds, the presence of coumarins was observed in the bark and leaves and steroids and triterpenes in the bark, which suggests that other compounds similar to those found in the roots that were not the target of our study may also be present in the bark and leaves of this species.

Regarding *T. avellanedae*, the leaf extract exhibited the highest content of flavonoids and showed a high efficiency for FIC and highest rate of growth inhibition of the HEp-2 cell line (88%). Suo et al. (2012) found the presence of naphthoquinones, furanonaphthoquinones, anthraquinones, benzoic acid derivatives, benzaldehyde derivatives, iridoids, coumarins, flavonoids, neolignan and benzoyl opioids in the bark of *T. avellanedae*; analyses of the four neolignan benzoyl opioids indicated anti-inflammatory activity, although the antioxidant activity was low. However, Park et al. (2003) reported antioxidant activity for the volatile compounds present in the bark of *T. avellanedae*, and Suo et al. (2013) reported that phenylpropanoid glycosides, which were also isolated from the bark of *T. avellanedae*, exhibited strong sequestration of free radicals. Of the three species examined in our study, *T. avellanedae* showed the lowest antioxidant capacity in the sequestration of free radicals and content of total phenols and condensed tannins and the highest content of flavonoids. Although the role of flavonoids as antioxidants is still controversial, the presence of these metabolites is associated with protection against the effects of reactive oxygen species (Choi et al., 2002).

With respect to the high levels of flavonoids, several studies have shown a correlation between the cytotoxic action in cell lines and presence of these compounds (Gil et al., 2009; Zhang et al., 2014), which would explain their effect on the HEp-2 cell line. The anticancer action of flavonoids is related to their effect on the sequestration of free radical, prevention of cancer progression, repair of DNA and stimulation of the immune system and their ability to effect the pathway that regulates growth and proliferation of cells and tumor formation (Pereira et al., 2009). Studies using hydroethanolic or ethanolic extracts from the bark of *T. avellanedae* (Warashina et al., 2005; Queiroz et al., 2008; Gómez Castellanos et al., 2009; Higa et al., 2011) have reported the presence of naphthoquinones, such as lapachol and β-lapachone, and their antitumor action in many cell lines (Cragg and Newman, 2005). In our study, naphthoquinones may also be associated with the antitumor effects observed in the HEp-2 cells. Studies using fractionated extracts, particularly the leaves of *T. avellanedae*, should be conducted to clarify whether this antitumor activity is related to the content of flavonoids, naphthoquinones or another class of metabolites.

Melo et al. (2010) studied the leaf methanol extract of *Handroanthus impetiginosus* (taxonomic synonym of *T. avellanedae*) that was collected in an area of Caatinga in Brazil and reported greater efficiency in the sequestering of free radicals and better cytotoxic activity for the NCI-H292 cell line compared with that of the HEp-2 cell line. Our results differ from those reported by Melo et al. (2010), and the high cytotoxic activity found for the HEp-2 cell line was likely caused by the differences between the extractor liquid used in the two studies and collection sites. The plants used in the present study were collected in the Cerrado region of Brazil, which has distinct ecogeographic characteristics from that of the Caatinga, which may have influenced the production of secondary metabolites (Gobbo-Neto and Lopes, 2007).

Conclusion

Considering all of the extracts and analyses, our study provides evidence for the anticancer activity of extracts from the bark and leaves of *A. humile* and the leaf of *T. avellanedae* and suggests that the growth inhibition of NCI-H292 and HT-29 cell lines may be related to the high content of condensed tannins, strong antioxidant activity in the sequestering of free radicals and strong FIC antioxidant activity, respectively. However, further studies with fractionated extracts and isolated compounds should be conducted to determine the complete antitumor therapeutic potential of these plants.

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Conflict of interest

The authors declare that there are no conflicts of interests regarding the publication of this paper.

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Podophyllotoxin content in rhizome and root samples of *Podophyllum hexandrum* Royle populations from Indian Himalayan region

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The podophyllotoxin content in rhizome and root samples of *Podophyllum hexandrum* Royle (with leaf morphological variants, that is, 1, 2 and 3L), an endangered perennial herb and a source of highly valued aryltetralin lignan, collected from 17 different populations (2800 to 3600 m asl) spread across Uttarakhand State of Indian Central Himalaya were analyzed by high performance liquid chromatography (HPLC). In general, podophyllotoxin content (on percent dry weight) of both rhizomes and roots varied significantly ($p < 0.05$) between the morphological variants. The podophyllotoxin content of rhizomes ranged from 0.012 to 5.480%; maximum and minimum levels were recorded in 2L (Kedarnath population) and 3L variants (Ghangria population), respectively. The mean podophyllotoxin content (population-wise) varied significantly ($p < 0.05$) between different populations; in general, a positive correlation ($p < 0.01$) was observed between podophyllotoxin content and increase in altitude; population-wise maximum (2.053%) and minimum (0.045%) levels were recorded in Kedarnath and Dayara-1 populations, respectively. The levels in root samples ranged from 0.021 to 5.800%, similar to those in the rhizomes. While maximum amount (5.800%) was estimated in 2L plants from Kedarnath population, minimum (0.021%) level was found in 2L plants from Ghangria population. The mean podophyllotoxin content across morphological variants (population-wise) was significantly ($p < 0.05$) higher in Kedarnath population (maximum: 2.090%); a positive correlation ($p < 0.01$) was found between the podophyllotoxin content and increase in altitude. Amongst all the morphological variants analyzed from different areas, 2L plants of Kedarnath population (highest altitude; 3600 m) exhibited maximum podophyllotoxin content, both in the rhizomes and roots. The observed chemo-diversity amongst morphological variants (leaf number) and populations could be used for selecting elites for multiplication and commercial and/or conservation purposes.

Key words: Alpine, cultivation, *Podophyllum hexandrum*, May apple, podophyllotoxin.

INTRODUCTION

Podophyllum hexandrum Royle (Syn. *P. emodi* Wale; Indian May apple; Family Podophyllaceae), a perennial herb distributed of high altitude (2000 to 4000 masl) areas of the Himalayan region, is a source of high value

compound of non-alkaloid nature, podophyllotoxin [Chemical name: 5,8,8a,9-Tetrahydro-9-hydroxy-5(3,4,5-trimethoxyphenyl) furo [3',4':6,7] naphtho [2,3,d]-1,3-dioxol-6 (5aH)-one; MW: 414.40]. It is the starting compound for the preparation of semi-synthetic compounds, namely, etoposide, etopophos and teniposide, and is useful in the treatment of lung cancer and refractory testicular cancer, stomach and pancreatic cancers, and myeloid leukemias (Van Uden et al., 1989; Stahelin and von Wartburg, 1991; Schacter, 1996; Ekstrom et al., 1998; Holm et al., 1998; Ajani et al., 1999; Lee and Xiao, 2005). It is also a precursor to CPH 82 (Reumacon) under trial for rheumatoid arthritis in Europe (Calstrom et al., 2000). In addition, podophyllotoxin has also been reported as a potent antiviral agent (Beutner and von Krogh, 1990).

The rhizomes of *P. hexandrum* are known to contain three times more podophyllotoxin than the American species *Podophyllum peltatum* (Fay and Ziegler, 1985). The reported total synthesis of podophyllotoxin has been found to be uneconomical. Further, while some attempts have been made for podophylloxin production using callus and suspension cultures of *P. peltatum* (Kadakade, 1982) and *P. hexandrum* (Chattopadhyay et al., 2001), respectively, large scale exploitation of natural populations continues to be the main source for pharmaceutical companies. In India, the compound is primarily obtained from rhizomes of *P. hexandrum* with concomitant decline in its natural populations. The species is currently in the negative list of exports of the Ministry of Commerce, Government of India (Lakhanpal, 1998), and also listed as “endangered” (Ved et al., 2003).

The presence of different lignans, particularly podophyllotoxin, in the rhizomes of *P. hexandrum* (Purohit et al., 1998; Sharma et al., 2000; Pandey et al., 2007; Nadeem et al., 2007; Naik et al., 2010; Kitchlu et al., 2011) and *P. peltatum* (Moraes et al., 2000; Canel et al., 2001; Zheljazkov et al., 2011) have been reported. Harvesting of this perrennating underground organ, for the analyses or commerce, adversely affects growth in subsequent years.

In the acute paucity of quality planting material of *P. hexandrum,* large scale production of propagules, either by conventional and/or biotechnological means (Nadeem et al., 2000) assumes importance for commercial plantations to meet the pharmaceutical demand. Identification of elites, in terms of high podophyllotoxin content, is a pre-requisite for meeting the aforementioned objectives. In view of this, *P. hexandrum* populations from alpine and sub alpine areas of Garhwal and Kumaun regions of Uttarakhand State in the Indian Central Himalaya (ICH) have been analyzed for podophyllotoxin content in the rhizomes and roots.

MATERIALS AND METHODS

Plant

The rhizome and root samples of *P. hexandrum* Royle [morphological variants with 1-leaf (1 L), 2-leaf (2L) and 3-leaf (3L)] were collected between mid-September to mid-October during 2000 from different geographical locations of Garhwal and Kumaun regions (2800 to 3600 m asl) of Uttarakhand in ICH (Table 1). It must be mentioned that the 4-leaf (4L) variant could not be located in any of the populations examined in this study. The botanical identity of the plants was confirmed and voucher specimens deposited in the G.B. Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Almora. The samples were sprinkled with a systemic fungicide, Bavistin (50% carbendazim w/w; BASF, Mumbai) and brought to the laboratory. The rhizome and roots were carefully separated, washed under running tap water to remove the adhering soil particles, rinsed with distilled water (thrice) and then allowed to air dry at room temperature. After drying, the samples were ground to a fine powder using mortar and pestle and packed in airtight polythene bags for storage at 4°C before analyses. The chemical analyses were carried out using composite samples prepared from three different plants of each of the seventeen populations examined.

Extraction and purification

Analyses for the estimation of podophyllotoxin were carried out following the method of Van Uden et al. (1989). The powdered rhizome and root samples (in triplicate; 200 mg) were extracted with 4 ml of 80% (v/v) aqueous methanol (MeOH). Following sonication (Branson, USA) for 1 h, the samples were allowed to stand at room temperature (24 h), the supernatant collected and the residue re-extracted with 2 ml of 80% (v/v) aqueous MeOH (24 h). Both supernatants were pooled and the final volume made up to 6 ml with 80% (v/v) aqueous MeOH. The pooled supernatant was then partitioned (thrice) with an equal volume of water and dichloromethane mixture (1:1, v/v; 6 ml each time), and the dichloromethane fractions were collected, dried in vacuo (30°C) in a rotatory film evaporator (Kinematica, Switzerland). The dried extracts were individually dissolved in 1.0 ml of HPLC grade MeOH and subjected to HPLC analyses.

High performance liquid chromatography (HPLC)

Purified samples (20 µl) were subjected to HPLC analyses (Kontron Instruments Ltd, Italy) on a RP-18 column (Lichrosorb, 250 × 4.6 mm id, 5 µm) eluted in an isocratic mode with MeOH:H_2O (60:40, v/v) at a flow rate of 1.0 ml/min. Detection was carried out at 290 nm using an online UV detector (Kontron). The podophyllotoxin content was estimated on the basis of peak area, using a standard curve made with known quantities of reference compound (Sigma Chemicals Co., USA) (Nadeem et al., 2007). The lowest limit of detection of podophyllotoxin under these experimental conditions was 25 ng.

RESULTS

HPLC analyses of rhizome samples of 17 different populations from the Garhwal and Kumaun regions

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revealed a wide variation in podophyllotoxin content among the morphological variants (1 to 3L) (Table 1). In general, the content varied significantly ($p < 0.05$) between the leaf variants, and the podophyllotoxin levels ranged from 0.012 to 5.480% (on dry weight basis) between populations. Maximum (5.480%) podophyllotoxin content was found in Kedarnath (2L), followed by Harkidun (2L) and Goi-2 (2L) populations, while the minimum (0.012%) level was found in Ghangria (3L) population. The podophyllotoxin content in rhizomes of 1, 2, and 3L plants ranged from 0.020 to 1.293%, 0.021 to 5.480% and 0.012 to 0.925%, respectively. The mean podophyllotoxin content in the rhizomes across leaf variants of seventeen populations exhibited significantly ($p < 0.05$) higher value for Kedarnath (2.053%, maximum), Murapara (1.072%) and Phurkia-2 (1.009%) populations, while the minimum (0.045%) level was found in Dayara-1 population (Table 1). The regression analysis suggests that the podophyllotoxin content of rhizomes was positively correlated with increase in the altitude ($y=0.002x – 5.6228$, $R^2=0.6346$; $p < 0.01$).

The podophyllotoxin content of root samples also varied significantly ($p < 0.05$) between morphological variants, and the levels ranged from 0.021 to 5.800% (on dry weight basis) among different populations (Table 1). Maximum (5.800%) podophyllotoxin content was found in Kedarnath (2L), followed by Dodital-2 (2 and 3L; 2.407 and 1.793%, respectively), while the minimum level (0.021%) was found in Ghangria (2L) populations. The content in roots of 1, 2, and 3L plants ranged from 0.025 to 1.377%, 0.021 to 5.800% and 0.028 to 1.793%, respectively. The mean podophyllotoxin content in the roots of the leaf morphological variants of seventeen populations exhibited significantly ($p < 0.05$) higher values for Kedarnath (2.090%, maximum), Dodital-2 (1.859%) and Auli (0.486%) populations, while the minimum (0.025%) level was found in Ghangria population (Table 1). The regression analysis suggests that the podophyllotoxin content was positively correlated with increase in the altitude ($y=0.0018x – 4.9423$, $R^2=0.4326$; $p < 0.01$).

DISCUSSION

It has been observed in the present investigation that, in general, samples collected from the higher altitudes (alpine regions) contained greater levels of podophyllotoxin in comparison to samples from the lower altitude (sub-alpine). However, in a few cases samples from the sub-alpine region also contained higher amounts of the active principle. It is interesting that amongst the morphological variants (1 to 3L), 2L plants of Kedarnath population contained maximum podophyllotoxin content, both in rhizome (5.480%) and root (5.800%) samples. Population-wise estimates indicated maximum podophyllotoxin content (rhizome-2.05%, root-2.09%) in Kedarnath plants; for both rhizomes and roots, a positive correlation was observed between the content and increase in the altitude. The plants (all the three morphological variants) from Kedarnath region, therefore, need particular attention.

Podophyllotoxin content of *P. hexandrum* rhizomes varies considerably, and values up to 8.26% have been reported (Purohit et al., 1998; Sharma et al., 2000). Levels in the range of 3.02 to 9.53% have been reported from 28 populations occurring at various altitudes (1570 to 4300 m) in the Northwest Himalaya (Naik et al., 2010). Similar levels were also reported for some populations collected from the trans-Himalayan region (Kitchlu et al., 2011). An earlier study from this laboratory found levels ranging from 0.36 to 1.08% in 8 populations (2740 to 3350 m) of the Kumaun region of ICH, exhibiting a positive correlation between the content and increasing altitude (Nadeem et al., 2007).

The occurrence of morphological variants with 1 to 4 leaves (1 to 4L plants) has been reported with an inverse relationship between podophyllotoxin content and the leaf number; the 4L plants contained lowest levels of the toxin as compared to 3, 2 and 1L plants (Purohit et al., 1998). In addition, *P. hexandrum* populations do exhibit marked variation in seed character, isozyme patterns and photosynthetic rates (Bhadula et al., 1996; Purohit et al., 1998).

In a recent study, podophyllotoxin content in the leaves and stems of morphological variants (1 to 3L plants) of *P. hexandrum* collected from seven populations (2800 to 3600 m) of the same region varied significantly ($p < 0.05$) between the variants, both in leaf and stem; the content ranged from 0.001 to 0.60%, and maximum content (0.60% in both leaf and stem) was found in 3L plants collected from Dodital (3100 m) population (Pandey et al., 2013). In another study, a maximum of 0.30% podophyllotoxin was reported in leaf samples collected from high altitude populations of Himachal Pradesh (Sharma, 2013). These values were, however, lower compared to contents up to 5.2% reported from leaf samples of *P. peltatum* (Moraes et al., 2005).

The variation in podophyllotoxin content in different populations of *P. peltatum* and *P. hexandrum* can be ascribed to genotypic differences (Bastos et al., 1996; Moraes et al., 2000; Nadeem et al., 2007; Naik et al., 2010). The age of the plant has been reported to influence the toxin content in *P. hexandrum* (Sharma et al., 2000; Pandey et al., 2007). In this investigation, analyses were carried out on samples collected from wild populations; hence, the exact age of the sampled plants could not be ascertained. It is possible that variations arise due to the presence of different chemotypes in natural populations and also on the method of extraction (Bastos et al., 1996; Canel et al., 2001). Further, both biotic and abiotic factors, including soil conditions affect lignan yield in *P. peltatum* (Moraes et al., 2005). While the morphological variants (1 to 3 L) of *P. hexandrum*

Table 1. Podophyllotoxin content in rhizome and root samples of morphological variants of *P. hexandrum* collected from seventeen populations across Garhwal and Kumaun regions of Uttarakhand in the Indian Central Himalaya.

Population (Place)	Altitude (m)	Plant type$^{\#}$	Podophyllotoxin content* (% of DW)			
			Rhizome	Population average$^{\$}$ (Rhizome)	Root	Population average$^{\$}$ (root)
		1 L	0.319±0.004^{a}		0.388±0.006^{a}	
Bharnala	2800	2 L	0.157±0.004^{b}	0.187±0.035^{cd}	0.380±0.005^{a}	0.334±0.021^{b}
		3 L	0.085±0.006^{c}		0.262±0.002^{b}	
		1 L	0.549±0.004^{a}		0.297±0.008^{b}	
Manjhi	2850	2 L	0.123±0.004^{b}	0.246±0.076^{cd}	0.260±0.003^{c}	0.392±0.057^{b}
		3 L	0.064±0.004^{c}		0.618±0.022^{a}	
		1 L	0.042±0.003^{b}		0.025±0.004^{a}	
Ghangria	2850	2 L	0.093±0.003^{a}	0.049±0.012^{d}	0.021±0.004^{a}	0.025±0.002^{b}
		3 L	0.012±0.002^{c}		0.030±0.003^{a}	
		1 L	0.044±0.003^{c}		0.044±0.003^{b}	
Goi-1	2850	2 L	0.278±0.003^{a}	0.188±0.036^{cd}	0.153±0.003^{a}	0.078±0.019^{b}
		3 L	0.243±0.003^{b}		0.037±0.003^{b}	
		1 L	1.293±0.004^{a}		0.193±0.004^{c}	
Murapara	2900	2 L	0.997±0.008^{b}	1.072±0.057^{b}	0.352±0.006^{a}	0.255±0.025^{b}
		3 L	0.925±0.030^{c}		0.221±0.006^{b}	
		1 L	0.020±0.001^{a}		0.041±0.002^{c}	
Dayara-1	2900	2 L	0.079±0.002^{a}	0.045±0.009^{d}	0.121±0.002^{a}	0.086±0.012^{a}
		3 L	0.036±0.002^{b}		0.097±0.002^{b}	
		1 L	0.656±0.004^{b}		0.320±0.004^{b}	
Goi-2	2950	2 L	1.652±0.012^{a}	0.804±0.227^{bcd}	0.491±0.004^{a}	0.280±0.068^{b}
		3 L	0.103±0.005^{c}		0.028±0.002^{c}	
		1 L	0.561±0.002^{a}		0.275±0.004^{a}	
Dayara-2	3000	2 L	0.021±0.002^{c}	0.206±0.089^{cd}	0.182±0.004^{c}	0.223±0.014^{b}
		3 L	0.037±0.002^{b}		0.213±0.004^{b}	
		1 L	0.046±0.003^{c}		0.055±0.003^{c}	
Garur Chatti	3000	2 L	0.359±0.004^{a}	0.208±0.045^{cd}	0.486±0.003^{a}	0.227±0.066^{b}
		3 L	0.219±0.003^{b}		0.140±0.002^{b}	
		1 L	0.047±0.002^{c}		0.077±0.003^{a}	
Auli	3000	2 L	0.137±0.004^{b}	0.142±0.028^{d}	0.137±0.004^{b}	0.486±0.190^{b}
		3 L	0.242±0.002^{a}		1.245±0.003^{c}	
		1 L	0.087±0.004^{c}		0.464±0.004^{b}	
Dodital-1	3000	2 L	0.322±0.004^{a}	0.193±0.034^{cd}	0.436±0.004^{c}	0.457±0.006^{b}
		3 L	0.170±0.003^{b}		0.470±0.003^{a}	
		1 L	0.544±0.003^{a}		0.639±0.003^{a}	
Khatyia^{+}	3000	2 L	0.465±0.008^{a}	0.409±0.049^{bcd}	0.321±0.004^{b}	0.359±0.076^{b}
		3 L	0.218±0.004^{a}		0.118±0.002^{c}	

Table 1. cont'd.

Phurkia-1[+]	3000	1 L	0.507±0.003[b]	0.512±0.048 [bcd]	0.296±0.005[b]	0.351±0.040[b]
		2 L	0.349±0.003[c]		0.509±0.005[a]	
		3 L	0.679±0.003[a]		0.246±0.004[c]	
Dodital-2	3100	1 L	0.118±0.002[c]	0.389±0.133[bcd]	1.377±0.349[c]	1.859±0.180[a]
		2 L	0.920±0.005[a]		2.407±0.007[a]	
		3 L	0.129±0.002[b]		1.793±0.013[ab]	
Phurkia-2[+]	3260	1 L	0.976±0.006[c]	1.009±0.116[b]	0.491±0.004[a]	0.338±0.063[b]
		2 L	1.425±0.014[a]		0.090±0.002[c]	
		3 L	0.626±0.006[b]		0.434±0.004[b]	
Harkidun	3400	1 L	0.284±0.007[c]	0.940±0.288[bc]	0.357±0.005[b]	0.425±0.088[b]
		2 L	2.087±0.012[a]		0.759±0.006[a]	
		3 L	0.449±0.006[b]		0.159±0.003[c]	
Kedarnath	3600	1 L	0.412±0.007[b]	2.053±0.857[a]	0.310±0.006[b]	2.090±0.928[a]
		2 L	5.480±0.045[a]		5.800±0.032[a]	
		3 L	0.268±0.005[c]		0.159±0.004[c]	

[#]1, 2 and 3 denote the number of leaves (L) in morphological variants; *Values are based on analyses of composite samples from 3 different plants of individual populations, and represent mean of 3 separate HPLC analyses; [$]average of 1L, 2L and 3L variants for each population; [+]From Kumaun region, the other 14 locations are from Garhwal region of Uttarakhand. All values represent mean± standard error. Mean values followed by the same letter(s) in a column are not significantly different ($p < 0.05$) based on DMRT.

from different populations contained varying levels of the toxin, the 2L plants from Kedarnath (highest altitude) contained maximum levels, both in the rhizomes and roots. These observations emphasize the need for selection of 'elites' based on higher toxin content. In view of the ever growing demand for podophyllotoxin and severe pressure on the natural populations, concerted efforts should now be directed towards selective propagation of elites. Propagation of *P. hexandrum*, both by conventional as well as *in vitro* methods reported from this laboratory (Nadeem et al., 2000), can be employed for mass scale multiplication of quality propagules for systematic cultivation in locations closer to their natural habitats (in alpine and sub-alpine regions) near village clusters. Modest efforts in this direction have been initiated and propagation of elites taken up and nurseries established in near natural habitats. Such steps would not only result in increased yields and assured supply, but would also pave the way towards conservation of this valuable medicinally important endangered species.

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Conflict of interest

Authors have not declared any conflict of interest.

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Full Length Research Paper

Alkaloid constitution of *Nerium oleander* using gas chromatography-mass spectroscopy (GC-MS)

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Oleander is an evergreen shrub or small tree from 5 to 25-ft tall containing gummy sticky sap in the dogbane family Apocynaceae. In this study, the alkaloid compounds of *Nerium oleander* (NO) have been evaluated. The chemical compositions of the leaf ethanol extract of *N. oleander* were investigated using gas chromatography-mass specroscopy (GC-MS). GC-MS analysis of *N. oleander* alkaloid leaf ethanol extract revealed the existence of the 5-hydrooxy methylfurfural, β-d-allopyranoside, methyle 6-dioxy-2-o methyl, cycloheptasiloxane, tetradecamethyle, cyclooctasiloxane, hexadecamethyl, cyclononasiloxane, octadecamethyl, cyclodecasiloxane, eicosamethyl, 2-cyclopenten-1-one, 2-hydroxy-3-methyl, 9.12.15-octadecatrienoic acid, 2,3bis[trimethylsilyl]oxy] propyl ester, octadecane, 3-ethyl-5-(2-ethylbutyl), 1-monolinoleoylglycerol trimethylsilyl ether, 1.1.3.3.5.5.7.7.9.9-decamethyl-9-(2-methyl propoxy)pentasiloxane, 2-cyclohexen-1-one, 4-(hydroxybutyle)-3.5.5-trimethyl, octasiloxane, 1.1.3.3.5.5.7.7.9.9.11.11.13.13.15.15-hexadecamethyl and 3-eicosene. Eight chemical alkaloids constituents have been identified from methanolic extract of the *Nerium oleander*.

Key words: Alkaloids, methanol, gas chromatography-mass spectroscopy (GC-MS) analysis, *Nerium oleander*.

INTRODUCTION

Nerium oleander (NO), common as wild plant in stony torrent beds or dry rocky valleys in foothills of Kurdistan up to altitude 800 m and as ornament plant throughout the country (Figure 1). The leaves contain neriin and oleandrin. Oleandrin is 3-glucoside-16-acyle derivative of gitoxigenin. They are poison to man, sheep, goat, cattle and horses. Root, bark and seeds contain the toxic principles neriodorin and karabin. The effects of poison are nausea, vomiting, colic dizziness, decreased pulse rate, irregular heart action, bloody diarrhea, and respiratory paralysis and death. Oleander is originally a Mediterranean and Asian plant and is widely distributed in the world, especially in tropical and subtropical regions. The plant is grown throughout Iran and is more common in eastern and southern provinces (Aslani, 2004). Oleander has long been known to be poisonous to animals and human beings. All parts of the plant either fresh or dried are toxic and contain cardiac glycosides,

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where the roots and seeds having the highest concentrations. Themost prominent of those glycosides are oleandrin and neriin (Aslani et al., 2007).

The main cardiac glycoside of *N. oleander* is oleandrin with a molecular formula of $C_{32}H_{48}O_9$ and a molecular mass of 576.3. The common oleander is one of most poisonous plants that have been shown to contain digitalis cardiac glycosides. Oleander is an idiom for plants of the *Nerium oleander* L, *Nerium indicum*, and *Nerium odorum* species. Common names include soland, lorier bol, rosebay, and rose laurel and kaner (Sazada et al., 2009). All parts of the oleander plant contain cardiac glycosides, including the roots and the smoke produced from burning, as heat does not inactivate the glycosides. The toxic components are the two potent cardiac glycosides; oleanderin and neriin, which can be isolated from all parts of the plant, both are very similar to the toxin of Foxglove (Shumaik et al., 1988; Cheeke, 1998; Singh et al., 1998). Oleandrin and neriin are the most potent of those glycosides (Longforad and Boor, 1996; Aslani et al., 2004). Oleander is one of the most toxic and lethal plants in birds. Accidental and/or experimental cases of oleander poisoning have been described in several species, including cattle (Aslani and Rezakhani, 2000; Soto-Blanco et al., 2006), sheep (Adam et al., 2002), goats (Aslani et al., 2007; Barbosa et al., 2008), camels (Kozikowski et al., 2009), horses (Hughes et al., 2002), donkeys (Smith et al., 2003), cats (Giuliano and Nebbia, 2004), dogs (Szabuniewicz et al., 1972), monkeys (Schwartz et al., 1974), turkeys, geese (Alfonso et al., 1994), canaries (Arai et al., 1992), ducks and budgerigars (Shropshire et al., 2003). The objective of this study was to determine the alkaloid compounds of *N. oleander*.

MATERIALS AND METHODS

Collection and preparation of plant

N. oleander leaves were collected from Hilla city Iraq. After collection of the required quantity of the plant material, it was then carefully segregated, cleaned and dried in shade to constant weight. The completely dried plant material free of moisture was powdered and sieved through a BSS Mesh No. 85 sieve and then stored in an airtight plastic container. Fresh areal parts of plant were collected for preparation of decoction.

Extraction and identification of alkaloids

The powdered leaves (2 g) were boiled in a water bath with 20 ml of 5% sulphuric acid in 50% ethanol. The mixture was cooled and filtered. A portion was reserved. Another portion of the filtrate was put in 100 ml of separating funnel and the solution was made alkaline by adding two drops of concentrated ammonia solution. Equal volume of chloroform was added and shaken gently to allow the layer to separate. The lower chloroform layer was run off into a second separating funnel. The ammoniac layer was reserved. The chloroform layer was extracted with two quantities each of 5 ml of dilute sulphuric acid. The various extracts were then used for the following test:

Wagner's test: To the filtrate in tube III, 1 ml of Wagner's reagent was added drop by drop. Formation of a reddish-brown precipitate indicates the presence of alkaloids (Evans, 2002).

Dragendoff's test: To the filtrate in test tube II, 1 ml of Dragendoff's reagent was added drop by drop. Formation of a reddish-brown precipitate indicates the presence of alkaloids (Evans, 2002).

Mayer's test: To the filtrate in test tube I, 1 ml of Mayer's reagent was added drop by drop. Formation of a greenish coloured or cream precipitate indicates the presence of alkaloids (Evans, 2002).

Gas chromatography-mass spectroscopy (GC-MS) analysis

GC-MS analysis of the methanol extract of *N. oleander* was carried out using a Clarus 500 Perkin-Elmer (Auto system XL) Gas Chromatograph equipped and coupled to a mass detector Turbo Mass Gold Perkin-Elmer Turbo Mass 5.1 spectrometer with an elite – 1 (100% Dimethyl poly siloxane), 30 m × 0.25 mm ID × 1 μm of capillary column. For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization system, and was operated in electron impact mode with ionization energy of 70 eV. The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period, the oven temperature rose up to 280°C, at the rate of an increase of 5°C min^{-1}, and maintained for 9 min. Helium gas (99.999%) was used as carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2 ml was employed (split ratio of 10:1). The injector temperature was maintained at 250°C, the ion-source temperature was 200°C, the oven temperature was programmed at 110°C (isothermal for 2 min), with an increase of 100°C min^{-1} to 200°C, then 5°C min^{-1} to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min and the total GC-MS running time was 36 min. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 45 to 450 (m/z). The mass detector used in this analysis was Turbo-Mass Gold-Perkin Elmer and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver 5.2 (Imad et al., 2014b; Imad et al., 2015b; Mohammed et al., 2015) .

RESULTS AND DISCUSSION

GC-MS analysis of alkaloid compound clearly showed the presence of fifteen compounds. The alkaloid compound, formula, molecular weight and exact mass are as shown in Table 1. The GC-MS chromatogram of the 15 peaks of the compounds detected are as shown in Figure 2. Chromatogram GC-MS analysis of the methanol extract of *N. oleander* showed the presence of fifteen major peaks and the components corresponding to the peaks were determined as follows. The first set up peaks were determined to be 5-hydrooxy methylfurfural, β-d-allopyranoside as shown in Figure 3. The second peaks indicated to be methyle 6-dioxy-2-o methyl (Figure 4). The next peaks was considered to be cycloheptasiloxane, tetradecamethyle, cyclooctasiloxane, hexadecamethyl, cyclononasiloxane, octadecamethyl, cyclodecasiloxane, eicosamethyl, 2-cyclopenten-1-one, 2-hydroxy-3-methyl, 9.12.15-octadecatrienoic acid 2,3bis

Table 1. Compounds present in the leaves extract of *N. oleander* using GC-MS analysis.

Alkaloid compound	RT (min)	Formula	Molecular Weight	Exact mass	Structure
2-Cyclopenten-1-one, 2-hydroxy-3-methyl	4.952	$C_6H_8O_2$	112	112.0524297	Figure 3
5-Hydrooxy methylfurfural	6.686	$C_6H_6O_3$	126	126.031694	Figure 4
β-d-allopyranoside, methyle 6-dioxy-2-o methyl	9.043	$C_8H_{16}O_5$	192	192.099773	Figure 5
2-Cyclohexen-1-one, 4-(hydroxybutyle)-3.5.5-trimethyl	11.904	$C_{13}H_{22}O_2$	210	210.16198	Figure 6
3-Eicosene	13.449	$C_{20}H_{40}$	280	280.313002	Figure 7
1-Monolinoleoylglycerol trimethylsilyl ether	15.938	$C_{27}H_{54}O_4Si_2$	498	498.356064	Figure 8
9.12.15-Octadecatrienoic acid,2,3bis[trimethylsilyl]oxy] propyl ester	17.329	$C_{27}H_{52}O_4Si_2$	496	496.340414	Figure 9
Octadecane, 3-ethyl-5-(2-ethylbutyl)	19.818	$C_{26}H_{54}$	366	366.422552	Figure 10

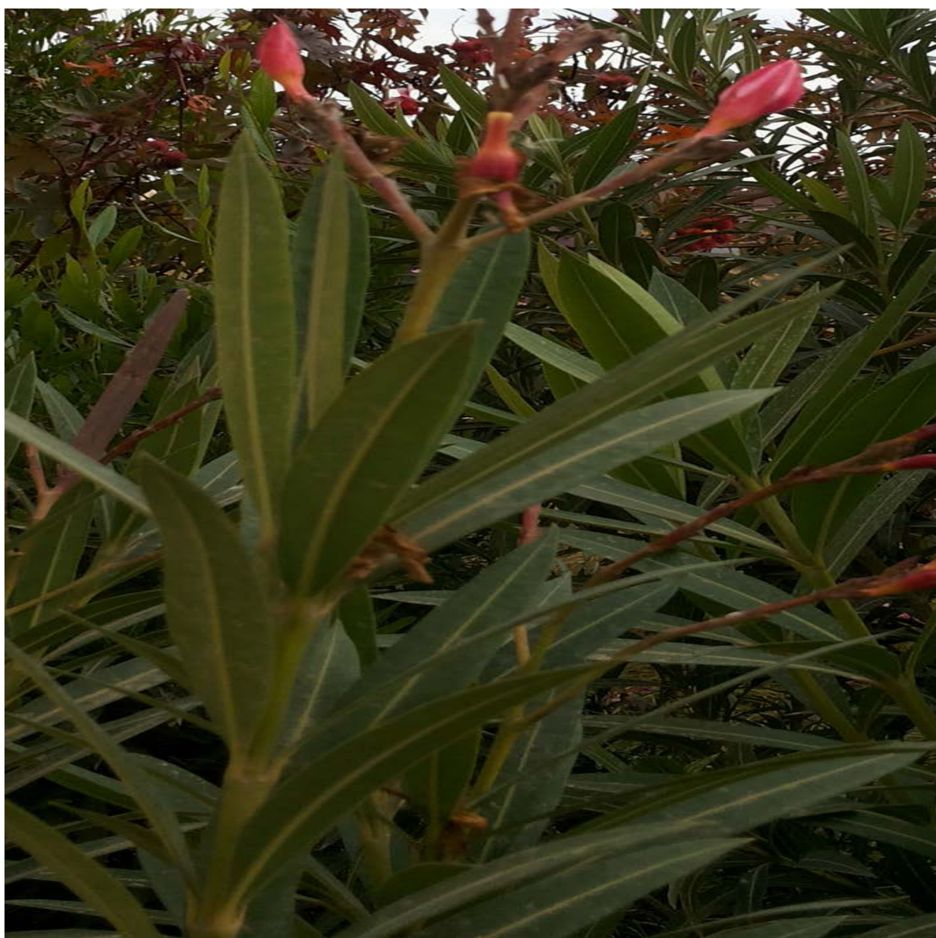

Figure 1. Plant of *Nerium oleander*.

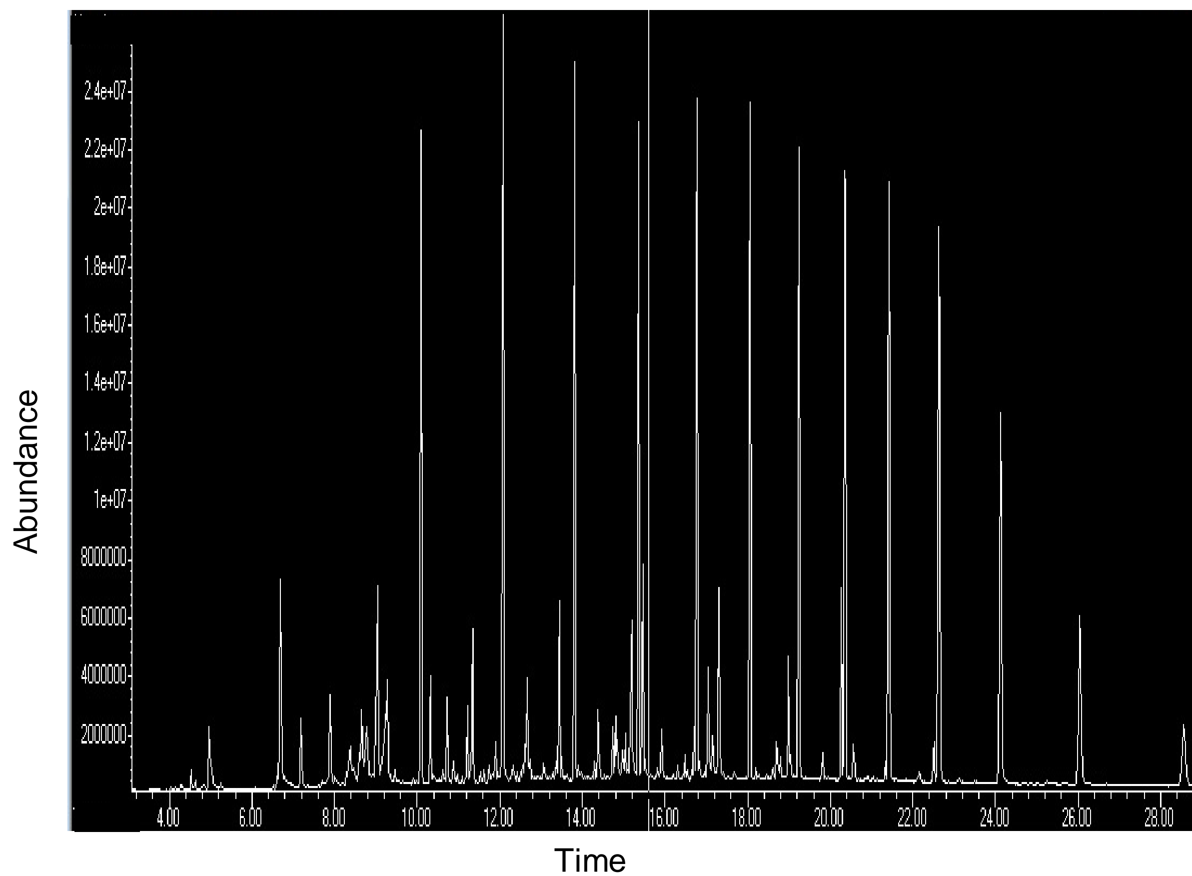

Figure 2. GC-MS Profile of leaves extract of *Nerium oleander.*

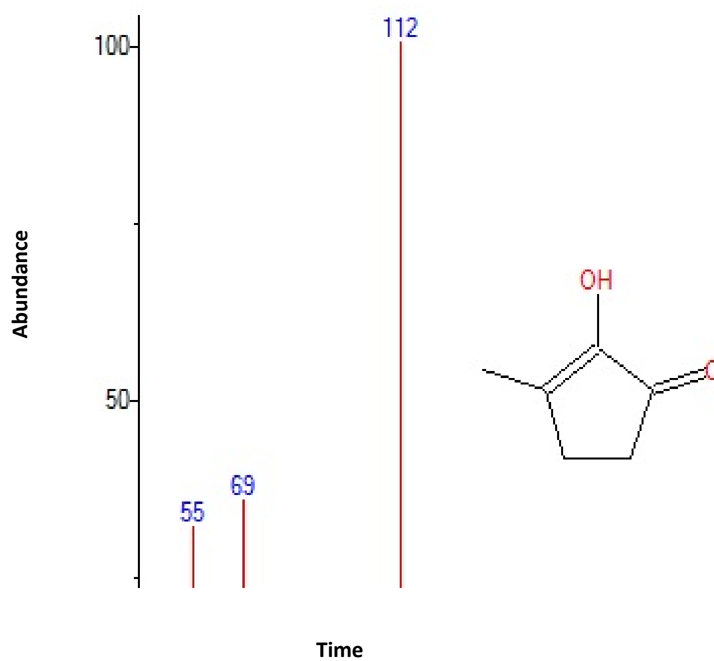

Figure 3. Structure of 2-Cyclopenten-1-one, 2-hydroxy-3-methyl present in the leaves extract of *N. oleander* using GC-MS analysis.

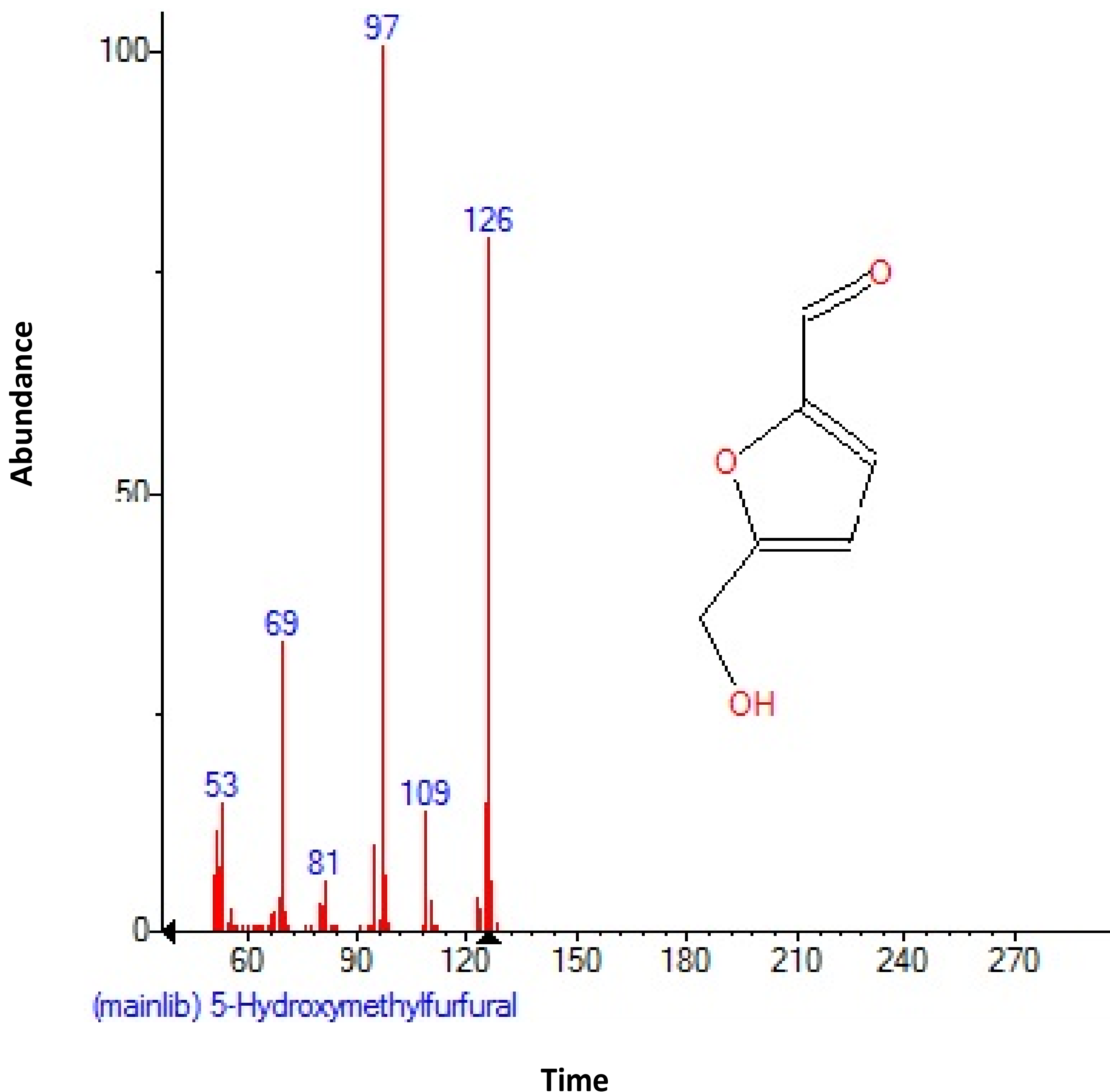

Figure 4. Structure of 5-Hydrooxy methylfurfural present in the leaves extract of *N. oleander* using GC-MS analysis.

[trimethylsilyl]oxy] propyl ester, octadecane, 3-ethyl-5-(2-ethylbutyl), 1-monolinoleoylglycerol trimethylsilyl ether, 1.1.3.3.5.5.7.7.9.9-decamethyl-9-(2-methyl propoxy)pentasiloxane, 2-cyclohexen-1-one, 4-(hydroxybutyle)-3.5.5-trimethyl, octasiloxane, 1.1.3.3.5.5.7.7.9.9.11.11.13.13.15.15-hexadecamethyl and 3-eicosene (Figures 5 to 10). Among the identified phytocompounds have the property of anti oxidant and antimicrobial activities (Kumar et al., 2001; Avci and Dik, 2014; John and Senthilkumar, 2005; Venkatesan et al., 2005; Santh, 2006). Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects. Continued further exploration of plant derived antimicrobials is needed today.

Conclusion

Fifteen chemical alkaloids constituents have been

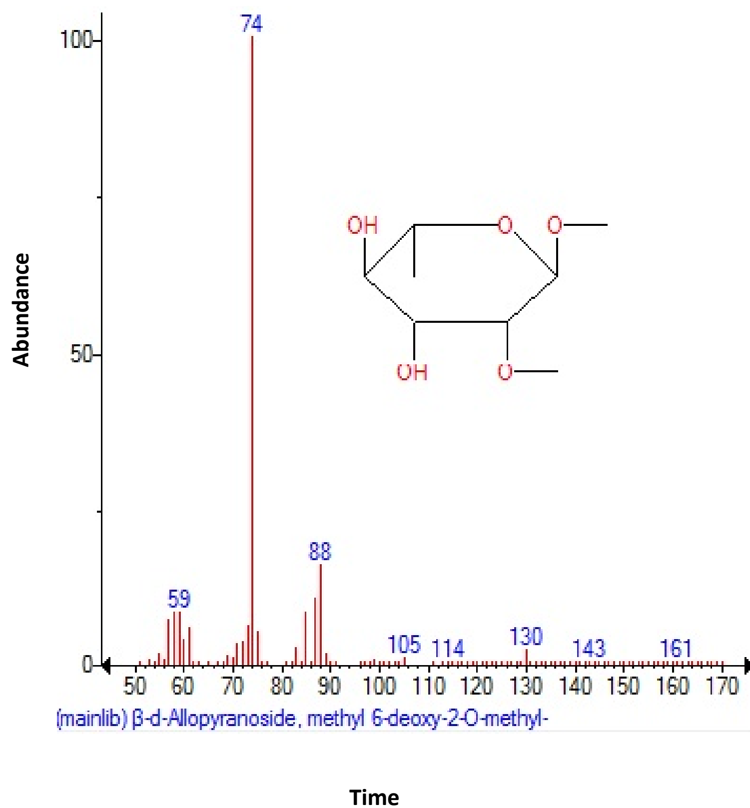

Figure 5. Structure of β-d-allopyranoside, methyle 6-dioxy-2-o methyl present in the leaves extract of *N. oleander* using GC-MS analysis.

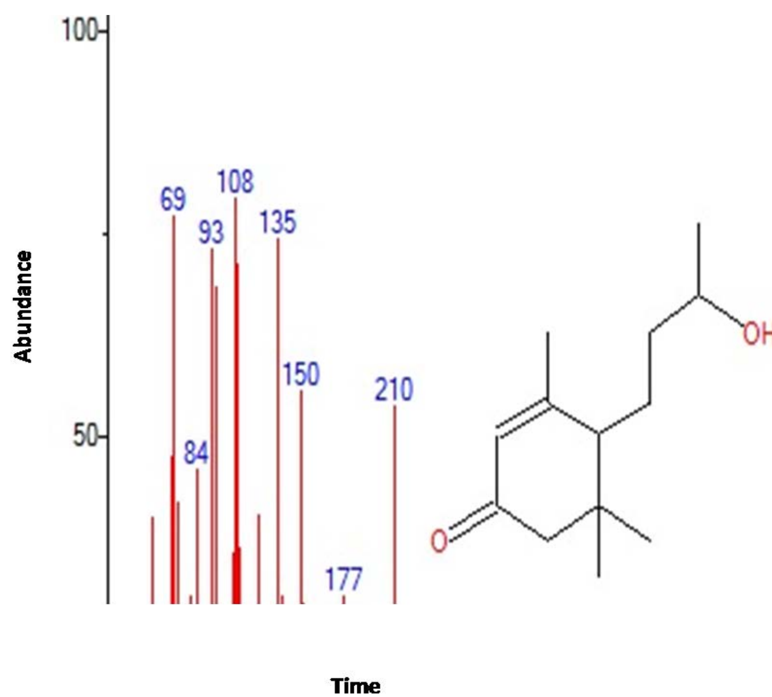

Figure 6. Structure of 2-Cyclohexen-1-one, 4-(hydroxybutyle)-3.5.5-trimethyl present in the leaves extract of *N. oleander* using GC-MS analysis.

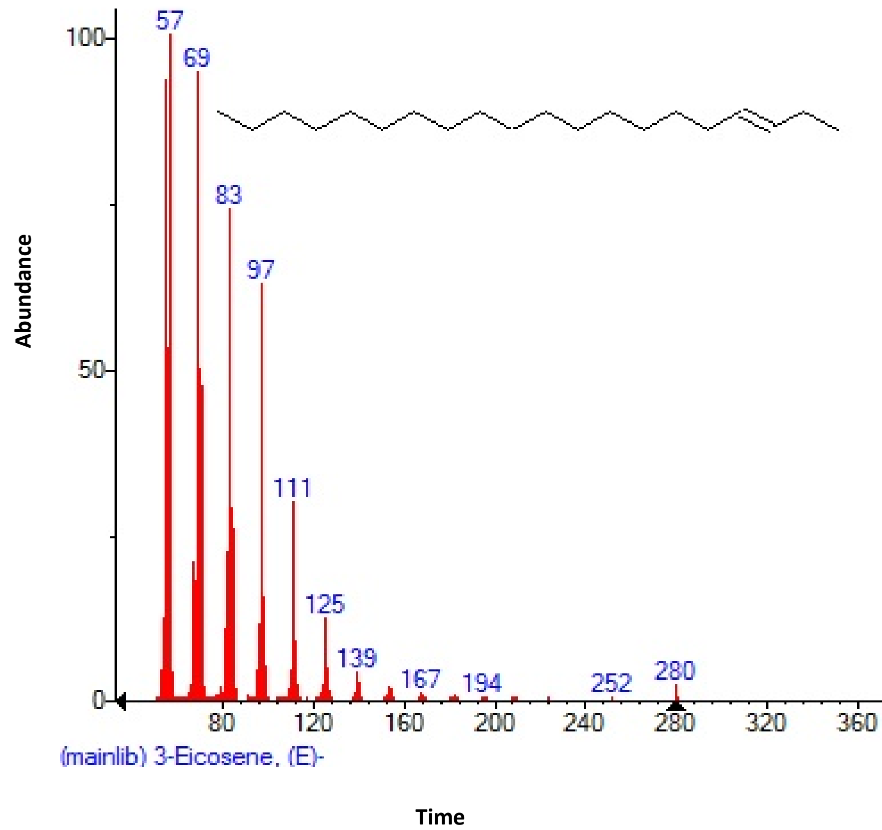

Figure 7. Structure of 3-Eicosene present in the leaves extract of *N. oleander* using GC-MS analysis.

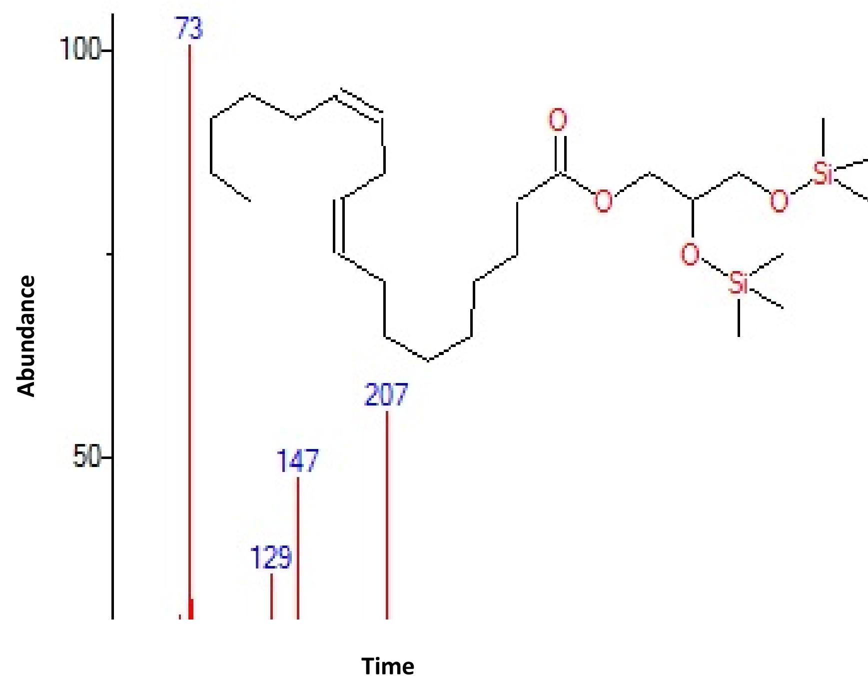

Figure 8. Structure of 1-Monolinoleoylglycerol trimethylsilyl ether present in the leaves extract of *N. oleander* using GC-MS analysis.

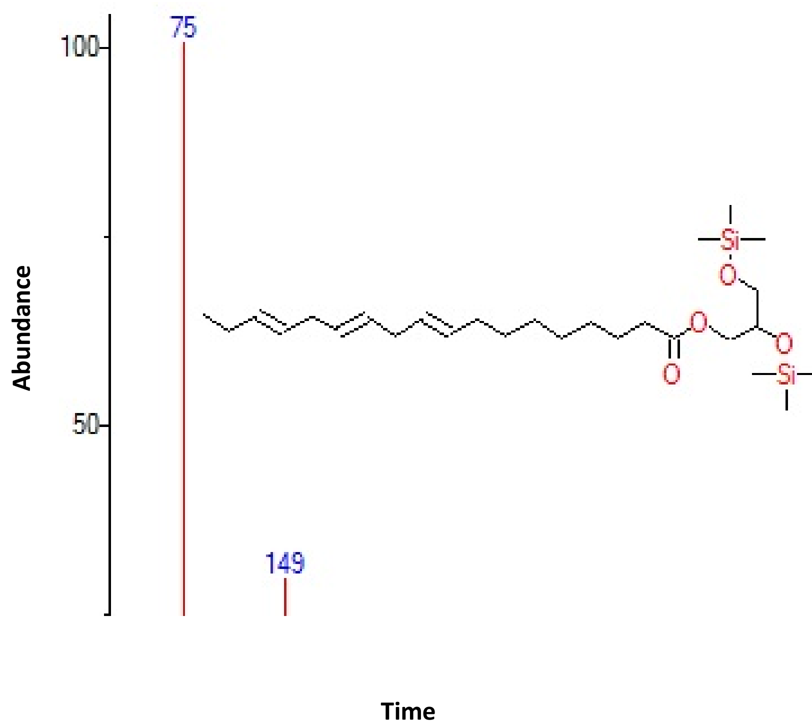

Figure 9. Structure of 9.12.15-Octadecatrienoic acid, 2,3 bis[trimethylsilyl]oxy] propyl ester present in the leaves extract of *N. oleander* using GC-MS analysis.

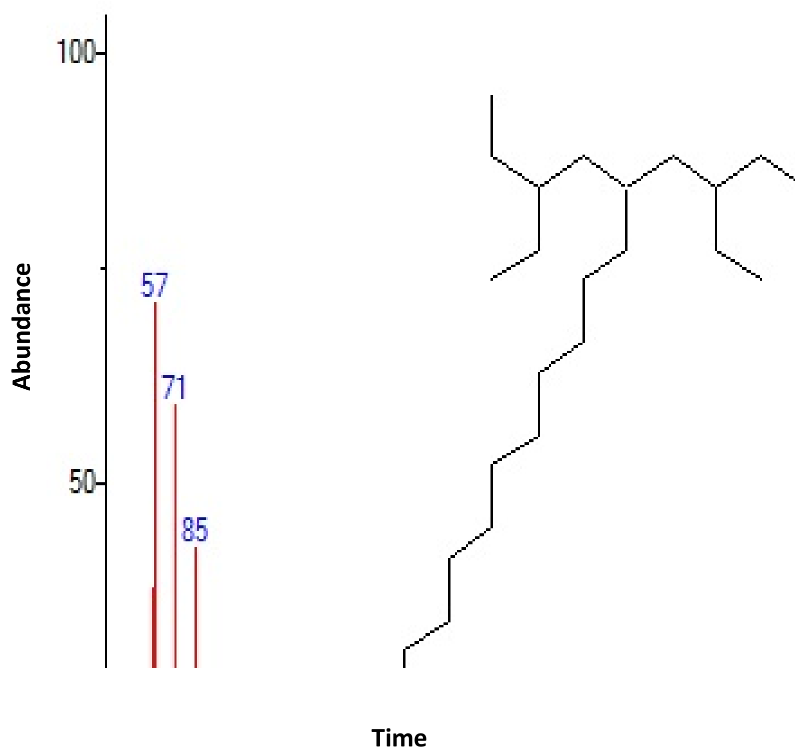

Figure 10. Structure of Octadecane, 3-ethyl-5-(2-ethylbutyl) present in the leaves extract of *N. oleander* using GC-MS analysis.

from methanolic extract of the Nerium oleander by Gas Chromatogram Mass spectrometry (GCMS).

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Conflict of interests

The author(s) have not declared any conflict of interests.

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