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Full Length Research Paper

Acceptability of culinary preparations based on different ground beef grades

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This work aims to evaluate the acceptability of culinary preparations formulated with different ground beef grades. Three standards of ground meat samples offered as special, first and second were purchased from local retail shops in Brazil and had its composition and collagen content determined. Culinary preparations were elaborated as meat sauce (braised); meatloaf (roasted) and hamburger patty (fried) evaluating influence of the ground meat kinds. Preparations had their composition assessed and sensory analysis by hedonic scale and preference by ranking test. Meat composition was influenced by commercial grade and retail source. There were higher protein concentration and moisture in special, prime and second ground meat, respectively. In an opposite way, lipid, collagen content and collagen ratio were higher at second, prime and special ground meat, respectively. The braised preparation had more acceptance when formulated with special or prime meat, whereas second grade led to greater acceptance in fried and roasted products. Expressions as "special" and "first" are used to assign quality to ground meats with greater protein and moisture, in addition to lower fat and collagen. The expression "second" is stated to meats distinguished from this standard. Acceptability of culinary preparations had independent quality grades designated by retail market and were mostly influenced by raw meat composition and particularities of the processes.

Key words: Collagen, lipids, meat, minced meat, nutrition, quality, sensorial analysis.

INTRODUCTION

Industry and retail market from several countries establish legal or informal quality standards to meat and meat products, in which classification and grading schemes for beef involves pricing, market and traders requirements and satisfaction of consumers. Recent Meat Standards Australia (MSA) research in Australia, Korea, Ireland, USA, Japan and South Africa showed that consumers across diverse cultures and nationalities have a

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remarkably similar view of beef eating quality (Polkinghorne and Thompson, 2010).

Ground Beef is defined as “chopped fresh and/or frozen beef with or without seasoning and without the addition of beef fat as such, shall not contain more than 30% fat, and shall not contain added water, phosphates, binders, or extenders” (FSIS, 2015). However, in Brazil there are different commercial kinds of ground meat, whose retailers use expressions like “special”, “prime” and “second” as its own quality grades, based on cut composition used as raw material (Grácia et al., 2010).

Factors like breed, age, cut composition and processing conditions impacts sensorial acceptance of meat derivatives (Abrahão et al., 2008). Work from Legako et al. (2015) showed that taste and juiciness of beef are more influenced by the cut origin than maturation stages. Moreover, Meinert et al. (2007) evidenced the positive effect over frying temperature on taste and odor of pork. The present work aims to evaluate the effect of culinary preparations based on different ground beef grades over composition and acceptability.

MATERIALS AND METHODS

Characterization of commercial ground beef presentations

Eleven establishments located in Curitiba, Paraná State, Brazil were evaluated as way of ground beef commercialization. Once the most frequent standards were identified as special (SPE), prime (PRI) and second (SEC), 300 g, in triplicate, of each commercial ground beef presentations were acquired in five retail shops and assessed for chemical composition.

Elaboration of culinary preparations utilizing ground meat with different composition grades

Culinary preparations were developed in a foodservice unit located in Federal University of Paraná, Brazil, with three experimental ground beef grades (SPE, PRI and SEC), similar to previously observed for commercial samples, processed. The three different compositions of ground meat were elaborated from the following deboned beef cuts: SPE (*Vastus lateralis* muscle removed from apparent fat and connective tissue); PRI (*Semimembranosus*, *Adductor femoris* and muscles *Gracilis* partially removed from same tissues); SEC (*Subscapularis*, *Supraspinatus*, *Infraspinatus* and *Triceps Brachii* without removal of any tissues). All cuts were minced (CAF, Brazil) with 4.7 mm and 3.1 mm plates and evaluated for chemical composition.

Three cooking methods were chosen for the culinary preparations: meat sauce (braise), meatloaf (roast) and hamburger patty (fry). Each preparation was formulated with 100 g from all three ground beef grades (SPE, PRI and SEC) and applied individually. Additionally to meat, all preparations had the following ingredients: chopped onions (10 g); parsley (0.5 g); garlic (0.2 g) and salt (0.7 g). Soybean oil (2 ml); chopped tomatoes (10 g) and powder beef stock (0.25 g) were used only in meat sauce. For meatloaf and hamburger patty, besides the cited ingredients, were also used eggs (5 g); wheat flour (10 g) and breadcrumbs (10 g).

Braising meat sauce was performed by mixing ingredients with raw meat, followed by heating for 25 min until internal temperature of 97°C. To the roasting process, samples were shaped in meatballs and put in oven (RATIONAL 40Gns, United Kingdom) for 17 min, until internal temperature reached 90°C. The frying procedure was realized with immersion of the hamburger patties in soybean oil at 180°C for 8 min (internal temperature of 87°C).

Proximate analysis

Analyses were performed on the commercial, experimental ground meat samples and culinary preparations. Moisture, lipid and as content were determined in triplicate (AOAC, 1995). Carbohydrates were obtained from the difference between 100 g of food and sum of the other components. The total collagen content was determined by measuring hydroxyproline (IAL, 2008), and proportion of collagen related to protein estimated by dividing collagen mean values by protein mean values and multiplied by 100 (Della Torre and Beraquet, 2005). All analyses were performed in triplicate. Caloric value (kcal) was estimated on a basis of 9 kcal/g for fat, 4.0 kcal/g for protein and 4 kcal/g for carbohydrates (Cuppari, 2005).

Sensorial analysis of culinary preparations

Culinary preparations were assessed by a 50-member untrained panel. The panel was randomly selected from users of the university foodservice. Were used 9-point hedonic scale and preference by ranking test, in which panelists chose between grades 1 (least preferred) to 4 (most preferred) (Meilgaard et al., 1999).

Statistical analysis

Differences among the means were compared using Tukey’s multiple range test using software R with significance level at 0.05. Sensorial data were analyzed with Newell and McFarlane table and level of 0.05 was chosen (IAL, 2008).

RESULTS AND DISCUSSION

Chemical characterization of commercial ground beef presentations

All of the 11 establishments evaluated had a few grade options of ground meat to distinguish their products. The most frequent way (5 out of 11 establishments) from retailers to show difference in their minced meat quality was a division in 3 grades: special (SPE); prime (PRI) and second (SEC). The different levels of quality observed on commercialization of beef indicated that when there is not an official designation, retailers offer products making their own quality standards. These results corroborate with early works that identified the use of quality grading based on regional particularities (Polkinghorne and Thompson, 2010).

Variations in meat composition for both commercial grades and retailers (Table 1) evidenced lack of standard in processing. Fat content had the wider variation between all parameters; one of the establishments (A) presented for SEC meat 15-fold more lipid than SPE

Table 1. Proximate analysis (%) of commercial and experimental ground beef.

Parameter	Grade	Establishments					Mean retail	Mean exp.
		A	B	C	D	E		
Fat	SPE	1.37±0.03^{b}	1.34±0.19^{b}	1.17±0.18^{b}	0.71±0.08^{a}	1.37±0.09^{b}	1.19±0.28	1.87±0.27
	PRI	2.75±0.20^{b}	8.97±0.47^{d}	1.16±0.04^{a}	1.44±0.38^{a}	4.32±0.08^{c}	3.73±3.18	6.45±0.65
	SEC	21.51±0.09^{e}	18.82±0.06^{d}	3.71±0.24^{a}	10.46±0.50^{c}	8.77±0.23^{b}	12.65±7.35	12.57±0.95
Protein	SPE	22.06±0.07^{bc}	22.56±0.39^{b}	21.53±0.12^{c}	23.77±0.27^{a}	22.25±0.15^{b}	22.43±0.83	20.38±0.42
	PRI	20.16±0.27^{b}	21.05±0.33^{b}	22.70±0.37^{a}	23.17±0.0^{a}	20.86±0.95^{b}	21.59±1.28	21.08±0.50
	SEC	14.28±0.38^{c}	20.03±0.59^{b}	21.30±0.04^{a}	22.20±0.47^{a}	21.60±0.45^{a}	19.88±3.23	18.03±0.10
Moisture	SPE	75.24±0.23^{a}	73.97±0.23^{b}	75.21^{a}±0.14^{a}	74.32±0.21^{b}	75.27±0.34^{a}	74.80±0.66	76.74±0.27
	PRI	75.38±0.54^{a}	68.14±0.46^{d}	74.78±0.23^{ab}	73.86±0.17^{bc}	73.24±0.37^{c}	73.08±2.88	72.26±0.09
	SEC	62.86±0.18^{d}	59.80±0.38^{e}	73.25±0.23^{a}	66.36±0.16^{c}	68.19±0.22^{b}	66.09±5.14	66.87±0.58
Ash	SPE	1.15±0.01^{ab}	1.22±0.02^{a}	1.16±0.06^{ab}	1.11±0.02^{b}	1.01±0.03^{c}	1.13±0.08	1.06±0.01
	PRI	0.98±0.09^{ab}	0.97±0.01^{ab}	1.05±0.03^{a}	0.94±0.02^{ab}	0.91±0.01^{b}	0.97±0.05	1.03±0.01
	SEC	0.84±0.05^{c}	0.80±0.04^{c}	1.01±0.02^{a}	0.94±0.01^{ab}	0.86±0.01^{bc}	0.89±0.08	0.86±0.01
Collagen	SPE	1.84±0.06^{d}	1.03±0.07^{b}	1.41±0.04^{c}	1.49±0.09^{c}	0.74±0.03^{a}	1.30±0.42	0.74±0.01
	PRI	3.12±0.31^{c}	2.91±0.19^{bc}	2.43±0.30^{ab}	3.52±0.14^{c}	2.13±0.11^{a}	2.82±0.55	1.08±0.12
	SEC	2.03±0.20^{a}	5.08±0.22^{c}	2.83±0.08^{b}	3.13±0.09^{b}	2.96±0.16^{b}	3.21±1.13	2.08±0.19
Col rel%	SPE	8.28±0.25^{d}	4.55±0.28^{b}	6.68±0.21^{c}	6.27±0.38^{c}	3.33±0.13^{a}	5.82±1.92	3.63±0.01
	PRI	16.74±1.68^{b}	13.84±0.91^{b}	10.68±1.33^{a}	15.18±0.58^{b}	10.22±0.54^{a}	13.33±2.83	5.12±0.58
	SEC	14.24±1.37^{a}	25.34±1.12^{b}	13.28±0.40^{a}	14.11±0.39^{a}	13.72±0.75^{a}	16.14±5.16	10.19±1.05

$^{(a, b, c)}$ Within the same columns, means having different superscripts are significantly different ($p < 0.05$). Exp: experimental; SPE: Special; PRI: Prime; SEC: Second. The results are given as the meant ± standard deviation.

meat. However, in retail shop C the same SEC meat showed fat content only 3-fold higher. The lipid content had more variation than observed by Flemming et al. (2003), who identified concentrations of 5.85 and 7.94% for ground meat found in grocery stores commercialized as PRI and SEC, respectively.

Lipid concentration means were increased as the order SPE<PRI<SEC, indicating that retailers relate fat content to reduced quality levels for meat named as “second quality”. The opposite order was observed for moisture, whereas SPE (74.80%) was followed by PRI (73.08%) and SEC (66.09%). The influence of lipid content over moisture was described by Pedrão et al. (2009) on Nelore (*Bos indicus*) cuts. *Longissimus dorsi m.* had 3.38% of fat content and 73.34% moisture, whereas *Rhoimboideus m.* showed higher lipid values (48.82%) and lower moisture (36.70%).

Samples exhibited average protein concentration of 22.43% (SPE), 21.59% (PRI) and 19.88% (SEC), a discrete variation in regards to influence of commercial grade or establishment. Only one sample (SEG) had protein mean under 20%. All

Table 2. Proximate analysis of culinary preparations elaborated with different ground meat grades.

Preparation	Standards	Parameter					
		Moisture (%)	Ash (%)	Fat (%)	Protein (%)	Carb. (%)	kcal/100 g
Braised meat	SPE	67.90±0.27	2.31±0.02	4.53±0.14	23.80±0.39	1.46	141.80
	PRI	61.82±0.28	2.28±0.04	9.81±0.16	24.62±0.44	1.47	192.65
	SEC	58.49±0.25	2.08±0.02	16.85±0.19	21.11±0.48	1.47	240.21
Roasted meatloaf	SPE	54.87 ±0.29	2.07±0.03	3.17 ±0.15	23.56 ±1.07	16.33	188.09
	PRI	52.30 ±0.27	2.09 ±0.01	5.18 ±0.28	25.13 ±0.87	15.30	208.34
	SEC	51.28 ±0.29	1.91 ±0.03	10.33±0.16	21.23 ±0.12	15.25	238.89
Fried hamburger patty	SPE	56.11 ±0.38	1.45 ±0.03	8.79 ±0.07	21.07 ±0.17	12.58	213.71
	PRI	55.33 ±0.36	1.50 ±0.05	9.45 ±0.21	21.70 ±0.07	12.02	219.65
	SEC	49.80 ±0.05	1.54 ±0.06	15.65±0.27	18.24 ±0.59	14.77	272.89

SPE: Special; PRI: Prime; SEC: Second. Carb: carbohydrates; kcal: kilocalories. The results are given as the mean ± standard deviation.

minced meats showed collagen increase by the order SPE<PRI<SEC, with means of 1.30, 2.82 and 3.21%, respectively. The values were within range observed for Della Torre and Beraquet (2005) who reported collagen concentrations between 1.0 to 4.9%, according to the commercial beef cut. Proportion of collagen related to protein also had an increase by the order SPE(5.82%)<PRI(13.33%)<SEG(16.14%). In one of the establishments (B), collagen proportion in SEC meat reached 25% of total protein. The increase of collagen content in SEC samples shows that retail also relate concentration of this protein to lower quality of ground meat. Ash values also varied among commercial grades and establishments, oscillating from 1.13% (SPE) to 0.89% (SEC) and were higher as the protein concentration increased (Table 1). These results were similar to previous works with beef cuts, alike *Rhomboideus* (Pedrão et al., 2009), *Serratus ventralis cervicis* and *Brachiocephalicus* (Marques, 2007), which showed average ash content of 1.03 and 0.99% respectively.

Chemical composition of culinary preparations formulated with different ground beef grades

Ground beef from all standards (SPE, PRI and SEC) elaborated in experimental scale showed (Table 2), as expected, similar composition observed on samples from retail shops (Table 1). Therefore, were able to be applied in culinary preparations.

The minced meat quality used as raw material was crucial to composition of culinary preparations. Above all formulations, the highest moisture was observed with samples processed with SPE kind. Protein content was lower in samples elaborated with SEG ground beef and alternated the most expressive means with meats SPE or PRI. Whereas, samples formulated with SEG minced meat showed higher fat content, followed by PRI and SPE products, respectively. The ash and carbohydrate levels varied according to ingredients used for the formulations (Table 2).

Caloric values, influenced by ground meat kind and cooking methods, had variations near 100%, from 141 kcal/100 g (braised meat/SPE) to 272.89 kcal/100 g (hamburger patty/SEC). Preparations showed an increase of caloric content proportional to lipid concentration in meat used as raw material. Hence, in all preparations, SPE had lower calories than PRI or SEC. Frying process used to hamburger patties elevated caloric values when compared to other cooking procedures (Table 2).

The temperatures and particularities from cooking methods also influenced composition of culinary preparations. Braised meat showed higher moisture among all preparations, especially in formulations with SPE meat. Hamburger patty composition reflected the soybean oil frying, resulting in higher lipid content, above all, in SEG preparations. Meatloaf, which was roasted in oven (dry air), showed reduced means for lipid and intermediate values for moisture in comparison to other preparations (Table 2).

Sensorial analysis

The raw material used in each culinary preparation promoted singular effect over sensorial analysis. Braised ground meat had highest acceptance on formulations with SPE or PRI, whilst SEC had lowest approval. On the opposite, meatloaf had highest acceptance when elaborated with SEC or PRI meat, and lowest scores with SPE. In hamburger patty, SEC promoted the best results, followed by SPE and PRI (Table 3). Hamburger patty had higher preference when formulated with SEC (124), followed by SPE (99) and PRI (83), the same result

Table 3. Hedonic scale and preference by ranking test of culinary preparations elaborated with different ground meat grades.

Presentation	Preparation					
	Braised meat		Roasted meatloaf		Fried hamburger patty	
	Hedonic	Preference by ranking	Hedonic	Preference by ranking	Hedonic	Preference by ranking
SPE	7.10[a]	113[a]	5.88[a]	90[a]	6.39[ab]	99[a]
PRI	6.92[a]	102[ab]	6.88[b]	107[ab]	5.82[a]	83[a]
SEC	5.50[b]	85[b]	6.90[b]	103[b]	6.86[b]	124[b]

(a, b, c) Within the same columns. means having different superscripts are significantly different ($p < 0.05$). SPE: Special; PRI: Prime; SEC: Second.

observed to hedonic scale test (Table 3). The increase of lipids in SEC had positive influence over fried or roasted preparations. Fat gives rise to palatability, flavor and tenderness in meat products (Keenan et al., 2014). On the other hand, lipid content in excess on diet may be a risk factor to several diseases (Kaliora and Dedoussi, 2007).

In general, as in braised meat, moisture had impact over acceptance of meat preparations. Moisture reduces muscular fibers compaction, improves texture (Youssef et al., 2007; Rocha Garcia et al., 2013) and juiciness of the meat products (Dubost et al., 2013). However, cooking method is able to reduce its levels (Table 2), taking in account the importance of lipid content over acceptability, as seen on meatloaf and hamburger patty. Early works from Badiani et al. (2002) showed that cooking procedures interferes on final composition values, lipid oxidation, cholesterol content and cooking loss, and may influence sensorial perception from panelists.

Collagen concentration and its proportion to protein increased as the relation SEC>PRI>SPE (Table 1). Even this protein is known to provide toughness, temperatures above 75°C promotes tenderness by gelation and degradation of connective tissue (Dubost et al., 2013), and might explain why higher concentrations of collagen had no influence on acceptance of meatloaf and hamburger patty with SEC meat. Nevertheless, cannot be neglected that collagen has a limited digestibility and lack of essential aminoacids, factors which may be considered on sensorial adjustment.

Conclusion

Meat market classifies meat with higher lipid and collagen content as inferior quality. However, sensorial analysis of culinary preparations had independent relationship between the quality grades designated by retail market and were mostly influenced by adjustments from meat composition to the particularities of the processes. Moisture favors sensory approval; nevertheless, cooking procedures that lower its level may give rise to the role of fat content over meat products.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Cytotoxic effect of *Plectranthus neochilus* extracts in head and neck carcinoma cell lines

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Following a tendency of studying the potential effects of plant extracts to cancer, this study aimed to evaluate *in vitro* the cytotoxic activity of *Plectranthus neochilus* (PN) extracts and its fractions in head and neck squamous cell carcinoma (HNSCC) cell lines and assess their tumor specificity. MTT assay was conducted with two HNSCC cell lines, FaDu (hypopharynx carcinoma) and SCC-25 (tongue carcinoma), one keratinocyte (HaCat) and one fibroblast (L929) cell line. Two PN leaf crude extracts, one ethanolic (E) and one hexanic (H), and their nine fractions were tested. A dose-response curve was performed with hexane PNH fraction and a tumor specificity index (TSI) was calculated. For all cell lines studied, almost all extracts and fractions resulted in cell viability lower than 50%. Hexane and methanol PNH fractions were exceptions, causing a significantly low viability in SCC-25 (17.16 and 34.53%, respectively), but higher than 50% in FaDu, HaCat and L929. The dose-response curve with hexane PNH fraction resulted in a CC_{50} of 540.9 µg/mL for FaDu, 550 µg/mL for L929, 762.1 µg/mL for HaCat and 274.2 µg/mL for SCC-25. The TSI L929/FaDu was 1.01, HaCat/FaDu was 1.40, L929/SCC-25 was 2.00 and HaCat/SCC-25 was 2.77. TSIs indicate its specificity for tongue carcinoma cells, when compared to fibroblasts and keratinocytes.

Key words: Head and neck, squamous cell carcinoma, extract, cytotoxicity, cell line.

INTRODUCTION

Cancer is one of the most common causes of morbidity and mortality today, being the cause of 8.2 million deaths in 2012 (World Health Organization (WHO), 2014). Such number is predicted to increase in the next years throughout the world, with an incidence of 14 million, as calculated in 2012, and estimated to rise up to 22 million in the next two decades (International Agency for Research on Cancer (IARC), 2012). Moreover, two thirds of all cancer diagnostics occur in low- and middle-income countries (WHO, 2014).

Head and neck cancer figures as the sixth most common type of cancer worldwide, being specially incident in the south and southeast of Asia, parts of Europe, and parts of South America (Warnakulasuriya, 2009). It is traditionally associated with tobacco and alcohol consumption, and more recently, the importance

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of human papillomavirus (HPV) as an etiological factor has been evidenced, mainly for oropharyngeal cancer (Pytynia et al., 2014; Thavaraj et al., 2011). According to the National Cancer Institute in the United States, the occurrence of 42,440 new head and neck cancer cases was estimated for 2014, as well as 8,390 deaths due to the disease (National Cancer Institute (NCI), 2014).

The treatment procedures depend on the stage of the disease, and surgery associated to radiotherapy and/or chemotherapy happens to be the treatment of choice in most situations. Obviously, each case ought to be analyzed independently, given that the morbidity of the surgical resection in the oral cavity is considerable and surgery must be avoided whenever possible (Belcher et al., 2014; Omura, 2014). Such idea instigates the search for new therapeutic options that are less deleterious to the patient's general health status than the surgery, radio and chemotherapy regimens prescribed currently.

The genus *Plectranthus* (Lamiaceae) comprises about 300 species distributed through the tropical and warm regions of the Old World, Africa, India and Australia (Rice et al., 2011). In Brazil, plants of this genus are widely used in folk medicine, mainly for digestive disturbances and liver complains (Bandeira et al., 2010; Lukhoba et al., 2006). The main phytochemical constituents of the genus *Plectranthus* are diterpenoids, essential oil and phenolics (Abdel-Mogib et al., 2002). However, concerning chemical composition for several species, little is still well known.

One of those, *Plectranthus neochilus*, called "boldinho", "boldo-da-folha-miúda" or "boldo-gambá" (Duarte and Lopes, 2007), beside digestive disturbance, is also used for pain, edema, skin infections and respiratory ailments (Caixeta et al., 2011; Madaleno, 2011; Moreira et al., 2002). In South Africa, an ethnomedicine survey revealed this species is used for treating respiratory infections (York et al., 2011). Little information may be found on its chemical composition. The essential oil composition is well reported, but can vary depending on several factors, for example, geographic differences, if the extraction occurs using fresh or dried plant material, and others (Caixeta et al., 2011; Lawal et al., 2010; Rosal et al., 2011). Essential oil from fresh leaves of *P. neochilus* from the southeastern region of Brazil presented β-caryophyllene, α−thujene and α-pinene as main compounds (Caixeta et al., 2011; Rosal et al., 2011), while a sample from South Africa showed citronellol and citronellyl formate as major compounds (Lawal et al., 2010). Hexane extract from leaves and stems furnished friedelin, fatty acid ester of α-amyrin, sitosterol and stigmasterol, while flavone cirsimaritin could be isolated from ethanol extract (Viana, 2011). In the same way, there are few references concerning biological activity of this species. The essential oil of leaves (100 µg/mL) presented activity against *Schistosoma mansoni*, killing 100% of adult worms (Caixeta et al., 2011). The essential oil causes the separation of male from female, deflecting the establishment of the infection. Cellular viability test using V79 cells (lung tissue of young male hamster) resulted in no cytotoxicity at doses lower than 200 µg/mL (Caixeta et al., 2011).

Essential oil from *P. neochilus*, presenting α-terpenyl acetate, α-athujene, β- caryophyllene, β-pinene, and α-pinene as major volatile components, was assayed with the thiobarbituric acid reactive substances (TBARS) test to evaluate the capacity for preventing lipid oxidation. As a result, a fairly anti-oxidant activity was observed only at high concentration (1.0 g/L), in comparison to α−tocopherol (Mota et al., 2014).

Methanol extract from leaves was tested against *Leishmania amazonensis* and *L. (L.) chagasi* and did not show any activity (Tempone et al., 2008). However, when tested against fluconazole-resistant *Candida krusei* (ATCC 6528) and the azole-susceptible *Candida parapsilosis* (ATCC 22019), it showed activity against *C. krusei* (EC_{50} = 20.51 µg/mL) (Tempone et al., 2008).

An ointment prepared with hydroethanol extract from leaves of *P. neochilus* as active component showed analgesic activity in female cats in post-surgery pain (Silva et al., 2013). Because *P. neochilus* is widely spread in Brazil, the aim of this study was to evaluate the potential cytotoxicity of crude extracts and fractions against head and neck squamous cell carcinoma cell lines and to assess the tumor specificity of these extracts by comparing their activity on cancer cells to their results on control cells.

MATERIALS AND METHODS

Plant

Aerial parts of *P. neochilus* were collected at Campus Universitario Darcy Ribeiro, Universidade de Brasília in January, 2011. A voucher species was deposited at Herbarium of Universidade de Brasilia (UB) (voucher number S. M. Gomes & P. Monteiro nº 913. After separation, leaves (979.0 g) were dried over temperature lower than 40°C and then submitted to extraction, by passive maceration technique, first using hexane, followed by ethanol. After solvent elimination under vacuum and temperature lower than 40°C, crude hexane extract (PNH, 5.1% yield) and crude ethanol extract (PNE, 7.5% yield) were obtained. Part of PNH (20.0 g) was submitted to silica gel 60 G Merck filtration (silica layer: high 5.1 cm, diameter 7.9 cm) furnishing 5 fractions: FHex (7.0 g); F (Hex:AcOEt) (12.0 g); FAcOEt (0.5 g); F (AcOEt:MeOH) (0.6 g); FMeOH (0.1 g). Part of PNE (6.0 g) was submitted to liquid-liquid partition and solvent extraction, resulting in 4 fractions: FrHex (1.0 g); $FrCH_2Cl_2$ (1.1 g); FrAcOEt (2.3 g); FrH2O (1.3 g). Crude extracts and fractions were submitted to cytotoxicity assay.

Cell lines and culture conditions

Human head and neck cancer cell lines, one of tongue squamous cell carcinoma (SCC-25) and another of hypopharyngeal carcinoma (FaDu) were used. A keratinocyte cell line (HaCat) and a fibroblast cell line (L929) were used as cell control. For the culture of SCC-25, cells were grown as monolayers in a mixture of Dulbecco's modified eagle medium (DMEM) and Ham's F12 in a proportion of 1:1, and supplemented with 10% fetal bovine serum and 1% antibiotics

Table 1. Evaluation of cell viability after treatment with *Plectranthus neochilus* crude extracts and their fractions.

Extracts and f ractions	Sample	No.	Viable cells (%)			
			FaDu	SCC-25	L929	HaCat
Plectranthus neochilus ethanolic crude extract	PNE	1	15.54	26.29	14.64	21.47
Plectranthus neochilus hexanic crude extract	PNH	2	6.77	31.44	16.38	6.12
Dichloromethane fraction from PNE	$FrCH_2Cl_2$	3	17.04	28.00	13.17	21.44
Hexane fraction from PNE	FrHex	4	4.59	39.41	11.28	5.02
Aqueous fraction from PNE	FrH2O	5	28.99	30.60	18.54	20.10
Ethyl acetate fraction from PNE	FrAcOEt	6	29.72	26.18	18.17	22.78
Ethyl acetate fraction from PNH	FAcOEt	7	9.66	10.13	7.23	6.98
Hexane fraction from PNH	FHex	8	84.72	17.16	69.95	82.29
Ethyl acetate:methanol (1:1) fraction from PNH	F(AcOEt:MeOH)	9	9.76	19.84	13.27	8.50
Hexane:ethyl acetate (1:1) fraction from PNH	F(Hex:AcOEt)	10	6.97	8.84	7.23	7.44
Methanol f raction f romPNH	FMeOH	11	63.45	34.53	52.36	62.77
Positive control	Cisplatin	-	50.38	36.55	31.24	36.51
Negative control	DMSO/Ethanol 2:3	-	100	100	100	100

Tongue carcinoma (SCC-25), Hypopharyngeal carcinoma (FaDu), Keratinocyte (HaCat) and Fibroblast (L929) cell lines. 1. *Plectranthus neochilus* ethanolic crude extract (PNE); 2. *Plectranthus neochilus*hexanic crude extract (PNH); 3. Dichloromethane fraction from PNE (FrCH2Cl2); 4. Hexane fraction fromPNE (FrHex); 5. Aqueous fraction from PNE (FrH2O); 6. Ethyl acetate fraction from PNE (FrAcOEt); 7.Ethyl acetate fraction from PNH (FAcOEt); 8. Hexane fraction from PNH (FHex); 9. Ethylacetate:methanol (1:1) fraction from PNH [F(AcOEt:MeOH)]; 10. Hexane:ethyl acetate (1:1) fraction fromPNH [F(Hex:AcOEt)]; 11. Methanol fraction from PNH (FMeOH).

(penicillin-streptomycin). HaCat, FaDu and L929 were cultured in DMEM with the same supplements described before. Cells were maintained at 37°C and 5% of CO_2. For all experiments, cells were detached with trypsin (0.25%)/EDTA (1 mM) solution. All cell culture reagents were purchased from Sigma-Aldrich (ET. Louis, MO).

Cell viability assay (MTT)

Cells were seeded at the density of 5×10^3 cells/well in a 96-well plate and then treated with *P. neochilus* extracts and its fractions (Table 1), at 500.0 µg/mL. After 24 h of treatment, cell viability was assessed by MTT assay. This test assesses the ability of mitochondrial enzymes of treated cells to convert tetrazolium salts (MTT) (3-[4,5- dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) in formazan, so only viable cells, or cells that have not undergone sufficient damage to reduce their mitochondrial activity, have the ability to accomplish this reduction. Then, the absorbance was measured and the values obtained with treated cells were compared to the values of cells treated with control. For this assay, 10.0 µL MTT solution (5 mg/mL) (Sigma) were added to each well, followed by incubation for 4 h at 37°C. After incubation the medium was discarded and formazan crystals were dissolved in 100 µL acidified isopropanol solution (25.0 mL isopropanol added of 104.0 µL HCl) and vortexed in low velocity for 15 min. Absorbance was measured at 570 nm in a Beckman Counter reader. Treatment with Cisplatin (50.0 µg/mL) was used as a positive control, and as negative control the solvent used with the extracts and fractions, which was dimethyl sulphoxide (DMSO): Ethanol (2:3). Experiments were carried out at least three independent times and were performed in triplicates.

Dose-response curve

HaCat, L-929, FaDu, and SCC-25 cells were seeded as described earlier for the cytotoxicity assay and incubated overnight in ideal conditions. Cells were then treated with serial dilutions of hexane PNH fraction in decreasing concentrations (1000.0, 750.0, 500.0, 250.0, 125.0 µg/mL) or vehicle control. After 24 h of treatment, MTT assay was performed as described. Graphpad Prisma 5.0 was used to define the 50% cytotoxic concentration (CC_{50}) of tested extracts. The IC_{50} values were used to calculate the tumor specificity index (TSI) for each treatment, calculated as the ratio of the IC_{50} of control cell lines and cancer cell lines, as proposed by Horii et al. (2012). The TSI equal to 1 means no selectivity between cell lines, TSI less than 1 means the treatment is more selective for control cell lines than for cancer cell lines and TSI greater than 1, it means that there is selectivity for the cancer cell line studied. The TSI was calculated according to the equation: TSI = (CC_{50} control cell) / (CC_{50} cancer cell) (Horii et al., 2012). L929 and HaCat were considered control cell lines and FaDu and SCC-25 were the cancer cell lines.

Statistical analyses of data

Statistical analysis was performed on the means of triplicates that resulted from at least three independent replications of all experiments. All data were analyzed using GraphPad Prisma 5.0. For cytotoxicity assay, one way analysis of variance (ANOVA) was used, with Tukey's multiple comparison test as a post-test. In dose response curve was used nonlinear regression, variable slope with log inhibitor *versus* response.

RESULTS AND DISCUSSION

The crude ethanol and hexane *P. neochilus* extracts (PNE and PNH, respectively) resulted in statistically significant viability reduction in all cell lines studied, which was always inferior to 50% of the cell viability compared with negative control (considered as 100% of viable cells), as seen in Figure 1 and Table 1. Such viability reduction was especially intense for the hypopharyngeal

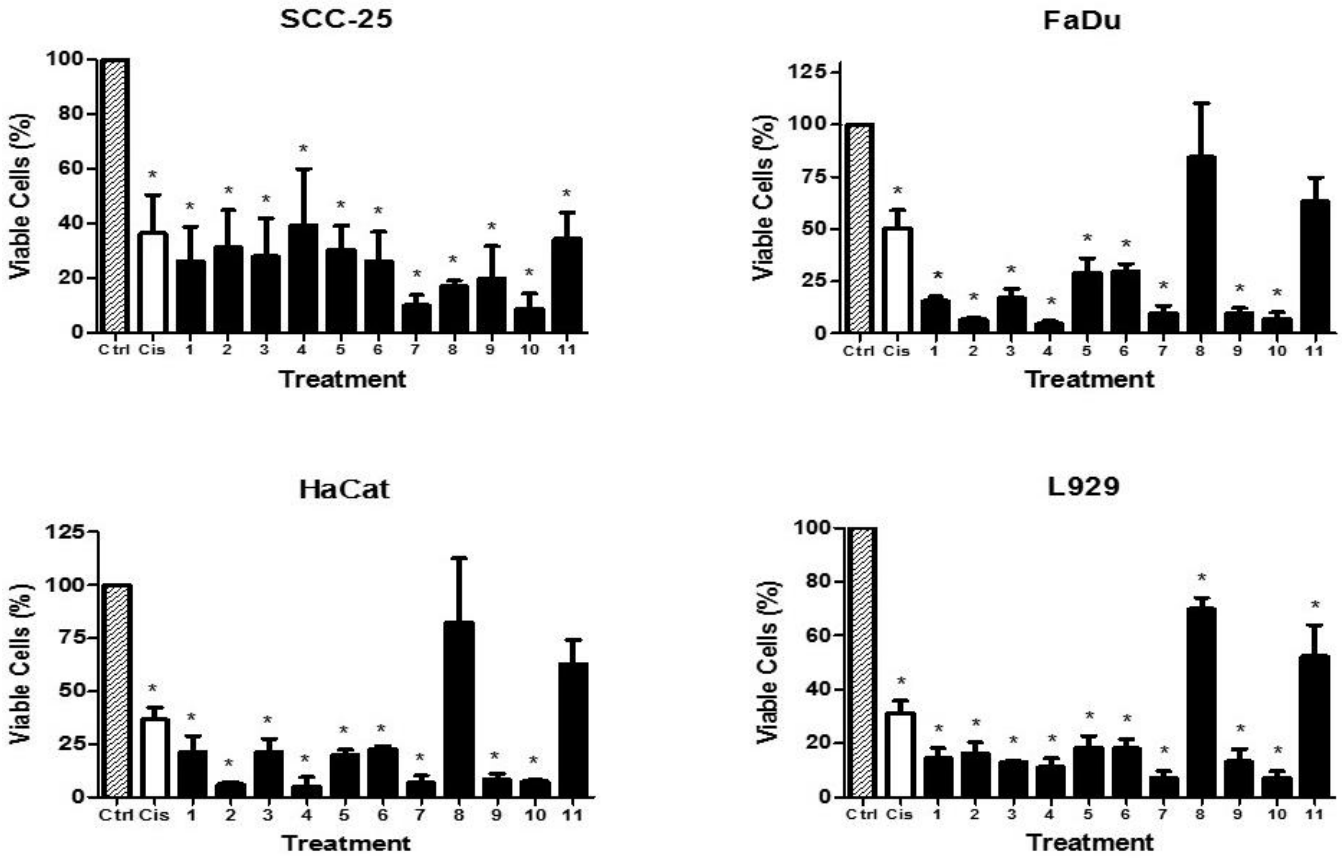

Figure 1. Cell viability after a 24 h-treatment with extracts and fractions. Tongue carcinoma (SCC-25), Hypopharyngeal carcinoma (FaDu), Keratinocyte (HaCat) and Fibroblast (L929) cell lines. Ctrl = Control (DMSO/Ethanol 2:3); Cis = Cisplatin (50 µg/mL); 1. *Plectranthus neochilus* ethanolic crude extract (PNE); 2. *Plectranthus neochilus* hexanic crude extract (PNH); 3. Dichloromethane fraction from PNE ($FrCH_2Cl_2$); 4. Hexane fraction from PNE (FrHex); 5. Aqueous fraction from PNE (FrH_2O); 6. Ethyl acetate fraction from PNE (FrAcOEt); 7. Ethyl acetate fraction from PNH (FAcOEt); 8. Hexane fraction from PNH (FHex); 9. Ethyl acetate:methanol (1:1) fraction from PNH [F(AcOEt:MeOH)]; 10. Hexane:ethyl acetate (1:1) fraction from PNH [F(Hex:AcOEt)]; 11. Methanol fraction from PNH (FMeOH). The results are representative of at least three independent experiments and show the mean±SEM.*p<0.05 *vs* control.

carcinoma cell line (FaDu), which resulted in a viability of 15.54% with PNE and 6.77% with PNH, for fibroblasts cells (L929), with a resultant viability of 14.64 and 16.38% for PNE and PNH, respectively, and for the keratinocytes cells (HaCat), with a viability of 21.47% for PNE and 6.12% for PNH. A viability of 26.29 and 31.44% could be observed in the tongue oral squamous cell carcinoma cell line (SCC-25) for PNE and PNH. Whenever compared to the positive treatment control, Cisplatin treatment with extracts was not considered statistically different, although the extracts seemed to be more cytotoxic than Cisplatin for all cell lines, given that they resulted in lower cell viability levels (Table 1).

The four PNE fractions (dichloromethane, hexane, aqueous and ethyl acetate fractions) and three of the PNH fractions (ethyl acetate, ethyl acetate:methanol and hexane:ethyl acetate fractions) were found to be highly cytotoxic to all tested cell lines, resulting in cell viability rates lower than 30% for FaDu (from 6 to 29%), HaCat (from 5 to 21%) and L-929 cells (from 7 to 18%), and lower than 40% for SCC-25 (from 8 to 39%) (Table 1). When compared to Cisplatin, these fractions were considered equally or more cytotoxic. Treatment with fractions resulted in a more intense cell cytotoxicity to HaCat and L929 than to tongue carcinoma cell line.

Different results might be observed for the hexane and methanol PNH fractions (FHex and FMeOH, respectively). FHex lead to a cell viability of 84.72% in FaDu, 69.95% in L929 and 82.29% in HaCat, while FMeOH fraction resulted in a viability of 63.45% with FaDu cells, 52.36% with L929 cells and 62.77% with HaCat cells. These cell viability rates were considerably higher than those observed after treatment with other fractions, the crude extracts and even the positive control. Interestingly, these fractions did not cause the tongue cancer (SCC-25) cell line such a small reduction in cell viability, when compared to Cisplatin or the other treatment regimens. In the tongue carcinoma cells, FHex

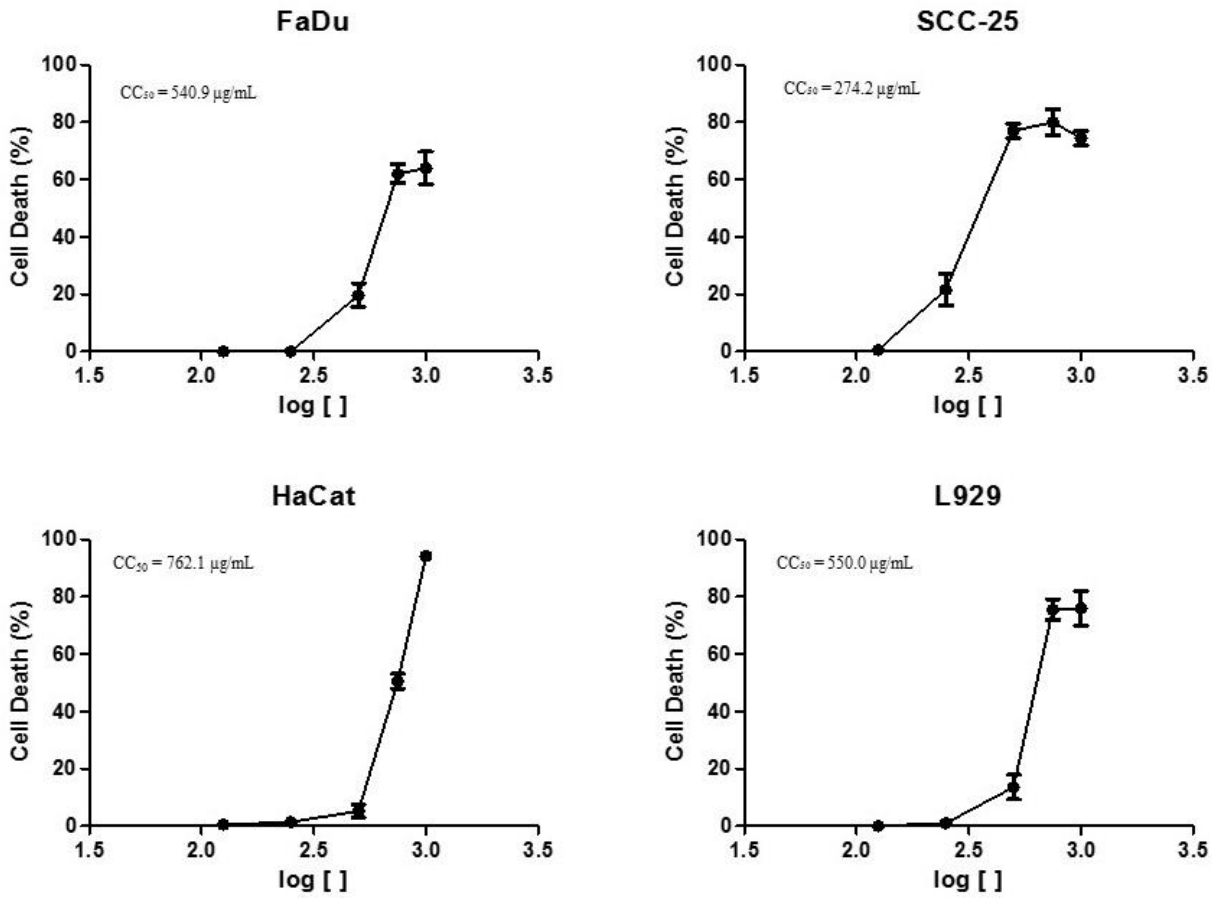

Figure 2. Hexane fraction from *Plectranthus neochilus* hexanic crude extracts (8. FHex) dose-response curves. CC50 = 50% Cytotoxic Concentration.Tongue carcinoma (SCC-25), Hypopharyngeal carcinoma (FaDu), Keratinocyte (HaCat) and Fibroblast (L929) cell lines.

caused a viability rate of 17.16% and a 34.53% rate was observed with FMeOH. Cisplatin resulted in a viability of 36.55% in this cell line.

Both FHex and FMeOH fractions were considered to be of special interest, given that they seemed to be highly cytotoxic to the tongue carcinoma cells and considerably less cytotoxic to the control fibroblast and keratinocyte cells. To further investigate and evaluate this potentially specific cytotoxic effect observed in this initial viability assay, dose-response curves were performed with the FHex fraction, for its cell viability result were lower with SCC-25 cells and higher with L929 and HaCat cells, when compared to the results observed with FMeOH fraction. The FHex fraction dose-response curves may be seen in Figure 2. A CC_{50} was calculated for each cell line. The doses of 540.9 µg/mL for FaDu, 550.0 µg/mL for L929 cells and 762.1 µg/mL for HaCat cells were calculated as the FHex CC_{50} in the respective cell lines. A smaller CC_{50}, 274.2 µg/mL, was found for SCC-25 cells, all in accordance with the results found in the initial cell viability assay.

The TSI was calculated on these values of CC_{50}. The TSI FaDu × L929 cells was 1.02 and the FaDu × HaCat was 1.41, which means that the fraction was not selective for the hypopharyngeal carcinoma cells, being equally cytotoxic to the cancer cells, fibroblasts and a little less toxic for keratinocytes. The TSI SCC-25 × L929 cells was 2.01 and the SCC-25 × HaCat cells was 2.78. These index values indicate a specificity of FHex for the tongue carcinoma cells (SCC-25), being the highest tumor specificity observed when SCC-25 and HaCat were analyzed. Similar results were already described in a study that found specific non-apoptotic cell death in oral squamous cancer cells induced by *Rhinacanthus nasutus* extracts (Horii et al., 2012). Interestingly, the specificity for cancerous cells was more evident when SCC-25 cells were taken into consideration, if compared to what could be observed with hypopharyngeal carcinoma cells (FaDu). Although both cell lines were derived from tumors that originate from a common cell type, the epithelial squamous cell, alterations and mutations in genes and molecular pathways vary in different tumors

(Chatelain et al., 2011). The genetic heterogeneity might justify the difference in results with SCC-25 and FaDu cell lines whenever they were submitted to the same treatment regime.

Conventional therapeutic approaches for HNSCC include surgery, radiotherapy and chemotherapy used alone or in combination. All therapies are associated with many adverse effects, such as post-surgical facial disfiguration or acute and chronic toxicities. Consequently, chemotherapy and radiotherapy reduce patient's quality of life. Many of these side effects are caused by the lack of selectivity of conventional therapies, which target both normal and cancerous cells. In this sense, the search for more new selective and effective therapies is highly required (Du et al., 2014).

Plants are major suppliers of substances active against cancer. For the first time, the cytotoxic activity of *P. neochilus* leaves extracts on HNSCC cell lines was reported. It is interesting to observe that, in general, the less polar fractions showed the most significant activity. A phytochemical analysis of PNH (unpublished results) characterized the presence of long chain fatty acids, triterpenes (friedelin and others), steroidal compounds (sitosterol, among others), diterpenes and sesquiterpenes. Caffeic acid derivatives can be found in PNE. Previous reports show that friedelin, sitosterol, some fatty acids and phenolic acids present cytotoxicity to several strains (Begin et al., 1988; Hoi et al., 2013; Matos et al., 2006; Ozcelik et al., 2011; Thao et al., 2010; Velasquez et al., 1993). Therefore, the presence of those compounds might explain the cytotoxicity observed.

Substances isolated from plants and fruits such as the aliphatic acetogenin constituents of avocado fruits show activity targeting EGFR/RAS/RAF/MEK/ERK1/2 pathway, which is considerably important in the carcinogenic process (D'Ambrosio et al., 2011). The lignin honokiol, isolated from magnolia species, induced stronger apoptotic activity in oral carcinoma cells when compared to 5-fluorouracil (Chen et al., 2011) and could be an alternative to chemotherapy treatments. Apoptotic death is desired in cancer treatment, given that necrosis is also associated with inflammation (Cho et al., 2011) and consequently induces more side effects in patients. In spite of the fact that these studies identified extracts and substances with high potential for cancer cytotoxicity and induction of apoptosis, none of them demonstrated specific activity against cancer cells.

Conclusion

The current study showed that the hexane PNH fraction presented specific toxicity for tongue carcinoma cells (SCC-25). For these reasons, further studies to investigate the cell death profile induced by hexane PNH fraction (FHex) are necessary. Moreover, a thorough investigation on the constitution of the extracts and fractions, the isolation of compounds and the study of their activity on a molecular level are also required, although the results obtained until now show that it could be a great alternative for a more specific treatment for tongue cancer.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Phytochemical screening and haematological studies of *Parquetina nigrescens* ethanol and chloroform leaves extracts in normal albino rats

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The effects of crude ethanol-chloroform extract of *Parquetina nigrescens* leaves on the haematological parameters of normal albino rats were investigated at dose of 50 and 100 mg/kg body weight (*b.w*). Acute toxicity study (LD_{50}) and phytochemical constituents of the extracts were also evaluated. Thirty (30) male Wistar rats were grouped into five (5) of 6 rats each (n = 6). Group A: Control; administered 2 ml of normal saline. The other groups were administered extracts viz: Group B - 50 mg/kg *b.w* of ethanol extract, Group C - 100 mg/kg *b.w* of ethanol extract, Group D - 50 mg/kg *b.w* of chloroform extract and Group E - 100 mg/kg *b.w* of chloroform extract. After 21 days of administration, the blood samples were collected for the analysis. The results revealed that, the ethanol and chloroform extracts caused significant ($p < 0.05$) increase in packed cell volume (PCV), red blood cell (RBC), haemoglobin, lymphocyte, neutrophil and eosinophil concentration at the doses administered compared with the rats in the control group. The rats administered 100 mg/kg *b.w* of chloroform extract showed significant ($p < 0.05$) increase in white blood cell differential compared with the rats in the control group, while the animals in the other treated groups showed non-significant ($p > 0.05$) decrease in white blood cell differential compared with animals in the control group. The monocyte and platelet concentration of the rats administered both extracts were found to have non-significant ($p > 0.05$) decrease at 50 and 100 mg/kg *b.w* of administration compared with animals in the control group. The results of the qualitative phytochemical analysis showed that the ethanol-chloroform extract tested positively to flavonoids, alkaloids, tannins, saponnin and reducing sugars while, chloroform extract tested positive to fat and oil and steroids. Acute toxicity and lethality studies on ethanol-chloroform extracts revealed an oral LD_{50} equal or more than 5000 mg/kg body weight in mice. It can be concluded that, the plant *Parquetina nigrescens* leaf has beneficial haematological and immunological properties in Wistar albino rats. It also revealed that *P. nigrescens* possessed erythropoietic potentials at minimal dose which lends support to its use in the treatment of anaemia.

Key words: Acute toxicity, phytochemical, immunological, anaemia, haematological.

INTRODUCTION

Plants and their derivatives play key roles in world health and have long been known to possess biological activity (Omoboyowa et al., 2013). The use of these plants by man for the treatment of various diseases has been in practice and is very popular in many developing countries of the world for over a long period of time (Gill, 1990; Idowu et al., 2009). This practice has gradually gained popularity in some parts of Europe and North America (Leese and William, 1994; Odeigah et al., 1999). In Africa, especially in the tropical areas, several factors such as poverty and illiteracy still militate against availability and accessibility of Western medical services. The need to have a strong, healthy immune system cannot be overemphasized in our present day society. Most illnesses such as AIDS and cancer are believed to be immune-related disorders (Idowu et al., 2009).

Medicinal plants possess therapeutic properties and despite the widespread use of modern medicine, herbal products are still in use in most developing countries of Africa and Asia for the management of ailments, *Parquetina nigrescens* happens to be one of such medicinal plants (Owoyele et al., 2011). *P. nigrescens* (Apocynaceae), a shrub found in equatorial West Africa, has been in traditional medicine practice for centuries. The parts of the plants used for traditional medicine include the leaves, roots and latex (Agbor and Odetola, 2005). It is a perennial with twinning stem and woody base shortly tapering 10 to 15 cm long, 6 to 8 cm broad with a smooth long stem on the leaves. The leaves have been reputed for treatment of helminthiasis (intestinal worm), wound and have sympathomimetic effect (Agbor and Odetola, 2005), while the roots are used for the management of rheumatism (Adeyemi, 1994). Over the years, *P. nigrescens* has been used as an ingredient in the medications for insanity (Iwu, 1993), as well as an aphrodisiac in East Africa. Other uses include the decoction of the stem bark been given as cardiac tonic while the leaf and root decoction have been used for the treatment of gonorrhoea and menstrual disorders (Iwu, 1993; Odetola et al., 2006). Research has shown that oral ingestion of medicinal compounds or drugs can alter the normal range of haematological parameters, these alterations could either be positive or negative (Ajagbonna et al., 1999). It has therefore become necessary to investigate the effect of ethanol-chloroform leaf extract of *P. nigrescens* on haematological parameters of rats *in vivo*, screening for selected phytochemical constituents of the present bioactive determination.

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade and supplied by sigma incorporated, US.

Plant

The leaves of *P. nigrescens* were collected from the metropolis of Ibadan, Nigeria. It was authenticated at the botany Department of University of Ibadan, Nigeria where a voucher specimen with voucher number V/No. 2002018 has been deposited. The leaves were air-dried at room temperature of 25 to 29°C after which it was ground. The pulverized leaves (1071 g) were macerated in 3 L mixture of chloroform and ethanol (2:1) for 48 h. The macerate was passed through Whatman No. 4 filter paper. The filtrate was shaken with 20% of distilled water to obtain two (2) layers. The upper layer (ethanol extract) was separated from the lower layer (chloroform extract) with a separating funnel. The two layers were concentrated with a rotatory evaporator and dried in a boiling water bath at 60°C. The weight of the extracts was taken after drying, the extract yields was obtained.

Phytochemical test

Basic qualitative phytochemical screening of the ethanol and chloroform extracts of the extract of the leaves sample was carried out by testing for the presence or absence of the following plant constituents: flavonoids, tannins, saponins, glycosides, terpenes, fat and oil, steroid, alkaloid, reducing sugar, phlabatannins and anthraquinone. The phytochemical analyses of the samples were carried out using the procedures outlined by Harborne (1989) and Trease and Evans (1989).

Experimental animal

Thirty male Wistar strain rats (200 to 240 g, initial weight) were used in the study. The animals were housed in wire mesh cages at the Department of Petroleum and Chemical Sciences, Tai Solarin University of Education, Ijagun, Ogun State, Nigeria. The animals were allowed to acclimatize for two weeks before commencement of the study. Food and water were provided *ad libitum*. Ethical approval was received from the College of Biological Sciences, Tai Solarin University of Education animal house committee.

Experimental design

The experiment lasted for 21 days and the animals were thus divided into five groups. Group A was the control and the rats were administered 2 ml of normal saline. The other groups (B, C, D and E) were the treatment groups. Groups B and C were administered 50 and 100 mg/kg *b.w* of ethanol extract, while groups D and E

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Table 1. Phytochemical constituents of the ethanol-chloroform extract of *P. nigrescens*.

Phytochemical constituents	Ethanol layer	Chloroform layer
Flavonoid	+	+
Alkaloid	+	+
Saponin	+	+
Tannins	+	+
Fat and oil	ND	+
Reducing sugar	+	+
Glycoside	ND	ND
Anthraquinone	+	+
Steroid	ND	+
Phlabatannin	ND	ND

Key: ND= Not Detected, + =Present.

were administered 50 and 100 mg/kg *b.w* of chloroform extract, respectively. All animals were sacrificed on day 21 after which blood samples were collected for the respective tests.

Acute toxicity and lethality *(LD_{50})* test

The acute toxicity and lethality of chloroform-ethanol extract of the *P. nigrescens* leaves extract was determined using the modified method of Lorke (1983). The test was divided into two stages. In stage one, eighteen (18) randomly selected adult mice were divided into six groups, three per group (n = 3) and received 10, 100 and 1000 mg/kg body weights (orally) of the ethanol and chloroform extracts, respectively and the signs of toxicity and number of death was observed for a period of 24 h.

After 24 h observation, the doses for the second phase were determined based on the outcome of the results of the first phase. Since there was zero death, a fresh batch of animals was used following the same procedure in phase I but with higher dose ranges of 1900, 2600 and 5000 mg/kg body weights of the extracts. The animals were also observed for 24 h for signs of toxicity and possible number of death. The LD_{50} was calculated as the geometric mean of the high non-lethal dose and lowest lethal dose (Lorke, 1983).

Haematological Indices

After 21 days of oral administration of the extracts, the animals were sacrificed and 2 ml of blood samples were collected by cardiac puncture for haematological analysis. The haematological parameters (Packed cell volume, haemoglobin concentration, red blood cell count, white blood cell count, lymphocyte count, neutrophil count, eosinophil count, monocyte count count and platelet count) were evaluated with an automated hematological analyzer systemex KX-21 (Japan).

Statistical analysis

The data obtained from the laboratory result of the tests were subjected to one way analysis of variance (ANOVA). Significant differences were observed at $p < 0.05$. The results were expressed as mean ± standard error of mean (SEM). These analyses were done using computer software known as statistical package for social sciences (SPSS), version 16.

RESULTS

Yield of the extracts

The yields of the extracts were calculated as 5.68 g (0.53%) and 15.85 g (1.48%) for ethanol and chloroform extracts, respectively.

Phytochemical test

The qualitative phytochemical compositions as observed in Table 1 showed presence of bioactive compounds such as flavonoids, alkaloids, saponnins, tannins and reducing sugar in the two extracts. The chloroform extract showed the presence of steroids and fat and oil. Anthraquinone was present in ethanol extract. The bioactive compounds found to be relatively absent in the extracts were glycosides and phlabatannins as shown in Table 1.

Acute toxicity and lethality (LD_{50}) test

Oral administration of up to 5000 mg/kg body weight of chloroform-ethanol extract of *P. nigrescens* leaves to mice caused no death in the two stages of the test. Thus, oral LD_{50} of the extract in mice was estimated to be greater than 5000 mg/kg body weight.

Effect of ethanol leaves extract of *Parquetina nigrescens* on haematological parameters in rats

Table 2 shows the results of the red blood cell count and some other haematological parameters in the experimental animals. The rats administered with 50 and 100 mg/kg *b.w* ethanol extract shows significant ($p < 0.05$) increase in packed cell volume, haemoglobin, red

Table 2. Effect of 21 days of oral administration of ethanol leaves extract of *Parquetina nigrescens* on haematological parameters in rats.

Parameter	Group A	Group B	Group C	Group D	Group E
PCV (%)	38.25 ± 0.63	41.75 ± 1.31*	42.50 ± 1.19*	41.00 ± 0.91*	40.50 ± 1.94*
Hb count (g/dl)	8.33 ± 0.31	9.20 ± 0.24*	9.30 ± 0.24*	8.43 ± 0.29*	8.65 ± 0.39*
RBC count (× 10^6 mm^3)	5.68 ± 0.35	7.64 ± 0.40*	8.52 ± 0.25*	7.49 ± 0.39*	8.32 ± 0.19*
WBC count (× 10^3 mm^3)	6.30 ± 0.41	6.28 ± 0.25	5.57 ± 0.50	5.35 ± 0.69	7.73 ± 0.14
Lymphocyte count (%)	64.25 ± 0.85	65.75 ± 4.35*	70.70 ± 2.66*	68.75 ± 1.79*	67.00 ± 2.42*
Neutrophil count (%)	27.25 ± 2.14	36.00 ± 3.37*	33.75 ± 4.53*	29.00 ± 2.71*	30.50 ± 1.94*
Eosinophil count (%)	1.0± 0.71	2.00 ± 0.71*	2.25 ± 0.48*	1.75 ± 0.25*	1.25 ± 0.25*
Monocyte count (%)	2.50 ± 0.29	2.25 ± 0.48	2.00 ± 0.71	1.75 ± 0.25	1.25 ± 0.25
Platelet count (× 10^4 mm^3)	9.80 ± 1.24	8.60 ± 1.50	8.63 ± 0.71	8.85 ± 1.47	8.63 ± 2.49

Key: Hb = Haemoglobin; PCV = Packed Cell Volume; WBC = White Blood Cells; RBC = Red Blood Cell *represents significant difference at $p< 0.05$. Group A: Control Group: 2ml of distilled water. Group B: Administered 50 mg/kg *b.w* of ethanol leave extract of *P. nigrescens*. Group C: Administered 100 mg/kg *b.w* of ethanol leave extract of *P. nigrescens*. Group D: Administered 50 mg/kg *b.w* of chloroform leave extract of *P. nigrescens*. Group E: Administered 100 mg/kg *b.w* of chloroform leave extract of *P. nigrescens*.

blood cell count, lymphocyte, neutrophil and eosinophil compared with the control group. The animals administered ethanol leave extract of *P. nigrescens* showed non-significant ($p > 0.05$) decrease in white blood cell differential compared with animals in the control group. The rats treated with 50 and 100 mg/kg *b.w* ethanol extract shows non-significant ($p > 0.05$) decrease in monocyte count and platelet count compared with the rats in the control group.

The results showed that, the red blood cell count and some other haematological parameters in the experimental animals treated with chloroform leave extract of *P. nigrescens*. The rats administered with 50 and 100 mg/kg *b.w* chloroform extract shows significant ($p < 0.05$) increase in packed cell volume, haemoglobin, red blood cell count, lymphocyte, neutrophil and eosinophil compared with the control group. The rats administered 100 mg/kg *b.w* of chloroform extract shows significant ($p < 0.05$) increase in white blood cell differential compared with the rats in the control group. The rats treated with 50 and 100 mg/kg *b.w* of chloroform extract shows non-significant ($p > 0.05$) decrease in monocyte count and platelet count compared with the rats in the control group.

DISCUSSION

Natural medicinal products have been used for the millennia for the treatment of multiple aliments although many have been superseded by conventional pharmaceutical approaches; there is currently a resurgence in interest in the use of natural products by the general public (Ghosh and Playford, 2003). The result of the haematological parameters observed in this study showed that the mean red blood cell count (RBC), haemoglobin, packed cell volume (PCV), lymphocyte, neutrophil and eosinophil concentration increased significantly ($p < 0.05$) at 50 and 100 mg/kg body weight (*b.w*) of administration compared with the control group. The mean white blood cell differential increases significantly ($p < 0.05$) at 100 mg/kg *b.w* of administration while the monocyte and platelet concentration was observed to have non-significant ($p > 0.05$) decrease at 50 and 100 mg/kg *b.w* of administration compared with the control group. As observed in this study, the chloroform and ethanol extract of *P. nigrescens* leaves had some positive effect on the haemopoietic system of the tested rats; this was manifested by an increase in RBC, Hb, PCV, lymphocytes, neutrophil and eosinophil concentrations following the administration of the medicinal plant extracts to the rats at varying doses. The raised PCV is an indication of haemoglobin concentration which may be due to increase RBC mass (Nwinuka et al., 2008). The observed increase in these haematological parameters may be due to the presence of erythropoietin-like principles in the extract which probably stimulated erythropoietin synthesis or release at low dose.

The significant increase in the value of lymphocytes, neutrophil and eosinophil in rats treated with the varying doses of the extracts compared with the rats in the control group and the significant ($p < 0.05$) increase in the white blood cell differential in rats administered 100 mg/kg *b.w* of chloroform extract compared with the control and other treated groups may suggest that, the chloroform and ethanol extracts of *P. nigrescens* leaves must have influenced the defence mechanism and immunity of the tested rats. Therefore, continuous exposure of the body systems of animals to the medicinal products (herbs) may cause lymphocytosis, which may then account for the use of this plant for medicinal purposes (Keenwe and Bekalo, 1996).

These results agree with the findings of Agbo and Odetola (2005) who investigated the aqueous leaf extract

of *P. nigrescens* on the erythrocyte indices. RBC count, haemoglobin concentration, haematocrit, reticulocyte and erythrocyte osmofragility were used as erythrocyte indices. It was observed that the aqueous leaf extract of *P. nigrescens* significantly ($p < 0.05$) increased the erythrocyte indices which were attributed to erythropoietic potential of *P. nigrescens*. The results obtained in this study is also in agreement with the findings of Owoyele et al. (2011) who observed increase in the erythrocyte indices in the study of haematological and biochemical studies of *P. nigrescens* root extract in albino rats.

Monocytes are known to originate in the bone marrow from a common myeloid progenitor that is shared with neutrophil and they are then released into the peripheral blood where they are circulated for several days before entering the tissues and replenishing the tissue macrophage populations (Siamon and Philip, 2005). Non-significant ($p > 0.05$) decrease in the monocytes concentration observed in the rats administered 50 and 100 mg/kg *b.w* of both extracts when compared with the control rats is an indication that chloroform and ethanol extracts of *P. nigrescens* may not have any adverse effect on the bone-marrow metabolism (Young and Maciejewski, 1997).

The specific bioactive constituent responsible for the hematologic properties of *P. nigrescens* leaves is yet to be identified. None of the several phytochemical constituents identified from the extracts has been reported to possess hematologic properties. The result of the qualitative phytochemical analysis observed in this study showed the presence of such bioactive compounds as flavonoids, alkaloids, saponnins, tannins and reducing sugars in the two extracts; steroids and fats and oil in the chloroform extract only while anthraquinone is detected in the ethanol extract only. The bioactive compounds detected such as tannins and flavonoids have been implicated in the treatment of diarrhoea while flavonoids and phenolic compounds are known to have antioxidant properties (Agbor and Odetola, 2001). This is an indication of a possible application of *P. nigrescens* in the treatment of other disease condition. However, the experimental data from this study is insufficient to directly ascribe the hematological properties to any of phytochemicals present in the twoextracts.

Acute toxicity test on the extract in mice estimated a high LD_{50} value of more than 5000 mg/kg body weight which suggests that the leaf may be generally regarded as safe with a remote risk of acute intoxication. The high degree of safety is also consistent with the report of Owoyele et al. (2011) and its popular use as herbs in the western part of Nigeria.

Conclusion

The chloroform and ethanol leaves extract of *P. nigrescens* possesses erythropoietic potentials and immunological properties at the varying doses used in this study and the overall results lend support to the folkloric use of the chloroform extract of *P. nigrescens* in the treatment of anaemia and in the enhancement of the immune system.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Design, synthesis and *in vitro* cytotoxic activity of Delavayin A: A cyclic hexapeptide

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Synthesis of new drug for anticancer activity has always been fascinating and interesting for researchers. Increase in the demand for new anticancer agents promotes the researchers for synthesizing new molecules with good activity on human cancer cell lines. Here design, synthesis and cytotoxic, antimicrobial activity of delavayin A is described. The structure of this synthesized compound was confirmed by (IR), 1H NMR, 13C NMR, Mass and elemental analysis. The synthesized compound was evaluated for *in vitro* cytotoxic activity by using Brine shrimp assay and on PC 3 and HL-60 cancer cell lines at Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Mumbai. The compound was also evaluated for antimicrobial activity by using minimum inhibitory concentration (MIC) technique. The synthesized cyclopeptide possessed moderate cytotoxic activity against cell lines and found to show moderate activity against Gram positive bacteria.

Key words: Cyclopeptide, cytotoxic, antimicrobial, *p*-nitro phenyl ester method.

INTRODUCTION

Development of new anticancer drugs from natural sources is fascinating and interesting for researchers working in the field of medicinal chemistry and drug development, as increase of resistance by cancer cells towards current anticancer drugs is a major issue. In recent years, synthesis of naturally occurring cyclic peptides as anticancer agents has attracted much attention of the researchers owing to their wide range of pharmacological activities shown by peptide molecules, which includes antimicrobial, anthelmintic, insecticidal, cytotoxic, anti-inflammatory activities (Chaudhary et al., 2012; Dahiya et al., 2010; 2011; Fernandez et al., 1992; Shinde et al., 2008; 2010). Keeping in view the significant biological activities exhibited by various cyclic peptides,

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as a part of ongoing study, an attempt was made towards the synthesis of a phenyl alanine rich cyclic hexapeptide, delavayin-A, cyclo (Gly-Ser-γ-hydroxy Ile-Phe-Phe-Ala-), which was isolated from the roots of *Stellaria delavayi* and belongs the family Cariophyllacea (Morta et al., 1997).

The synthesis was carried out by using solution phase technique and the synthesized compound then subjected to cytotoxic screening against PC-3 and HL-60 human tumor cell lines. The synthesized molecule was also evaluated for antimicrobial activity. The synthesized cyclopeptide possessed moderate cytotoxic activity when tested against PC 3 and HL-60 cancer cell lines comparable with the standard drug adriamycin. The compound had also shown prominent activity against Gram positive bacteria's and moderate activity against pathogenic fungi in comparison with benzyl penicillin and fluconazole, as standard.

MATERIALS AND METHODS

General

All L-amino acids, di-*tert* butyldicarbonate (Boc_2O), diisopropylcarbodiimide (DIPC), trifluoroacetic acid (TFA), triethylamine (TEA), pyridine and *N*-methylmorpholine (NMM) were procured from Spectrochem Limited (Mumbai, India). Melting points of all intermediates and final compound were determined by using digital melting point apparatus, make SYSTRONIC. The IR spectra were recorded by using KBr pellets or utilizing chloroform and NaCl cells on JASCO 4100 FTIR spectrophotometer. 1H NMR and 13C NMR spectra were recorded on Bruker AC NMR spectrometer using dimethyl sulphoxide (DMSO) as a solvent. The mass spectrum of the cyclopeptide was recorded at 70 eV on JMS-DX 303 Mass spectrometer.

In order to carry out the total synthesis of cyclopeptide, cyclo(Gly-Ser-γ-hydroxy Ile-Phe-Phe-Ala-) 12, it was disconnected into three dipeptide units, Boc-Gly-Ser-OMe 7, Boc- γ-hydroxy Ile-Phe -OMe 8, Boc-Phe-Ala-OMe 9 (Scheme 1). The required dipeptides were synthesized by coupling Boc amino acids 1,3 and 5 with respective amino acid methyl ester hydrochlorides 2,4 and 6 using DIPC, $CHCl_3$ and N-methyl morpholine according to Bondanszky procedure. The Boc-group of the dipeptide 8 was removed by using trifluoroacetic acid (TFA), 10% $NaHCO_3$ and the ester group of dipeptide 7 was removed by using LiOH. The deprotected units were then coupled to get a tetrapeptide Boc- Gly-Ser-γ-hydroxy Ile-Phe OMe 10. The hydroxyl group was introduced into isoleucine by using standard procedure (Pleissner and Wimmer, 2011) (Appendix 1). Similarly, the dipeptide 9 was coupled with tetrapeptide 10 after appropriate deprotection to get a linear hexapeptide 11 by using DIPC, N-methyl morpholine and chloroform. Finally by using p-nitrophenyl ester method (Bodanszky and Bodanszky, 1984) cyclisation of linear hexapeptide was carried out to get the titled compound 12. All intermediates and final product were recrystallized from $CHCl_3$.

The IR spectrum showed presence of -CO-NH- moiety in the cyclized product. The NMR spectrum of cyclized product clearly indicates the presence of all respective amino acid moieties. Furthermore, the mass spectrum of this cyclic hexapeptide showed a molecular ion peak at m/z 640.3. The synthesized cyclic hexapeptide was screened for cytotoxic activity by using Brine shrimp assay and also against human tumor cell lines by comparing with standard adriamycin at ACTREC Mumbai. The synthesized compound was also evaluated for antimicrobial activity by using tube dilution technique against four bacterial species and two fungal strains. Benzyl penicillin and fluconazole were used as standards for antibacterial and antifungal activity, respectively.

General method for preparation of Di/Tetra/linear hexapeptide

L-Amino acid methyl ester hydrochloride/dipeptide methyl ester/tetra peptide methyl ester (10 mmol) was added to chloroform (CCl_3, 20 ml). To the resulting solution, at 0°C, TEA (2.8 ml, 20 mmol) was added and the reaction mixture was allow to stir for 15 min. Boc-L-amino acid/Boc dipeptide/Boc tetrapeptide (10 mmol) in chloroform (20 ml) and DIPC (10 mmol) were added while stirring. The reaction mixture was allowed to stir for 24 h, filtered and the residue was washed with chloroform (30 ml) and added to the filtrate. Washing of the filtrate was done by using 5% $NaHCO_3$ and saturated NaCl solutions and the resultant organic layer was dried out by anhydrous Na_2SO_4, filtered and evaporated in vacuum. Recrystallization of the crude product was done from a mixture of chloroform and petroleum ether (b. p. 40 to 60°C). By using aforementioned procedure, compounds 1 to 7 were synthesized.

Method for cyclization of linear hexapeptide

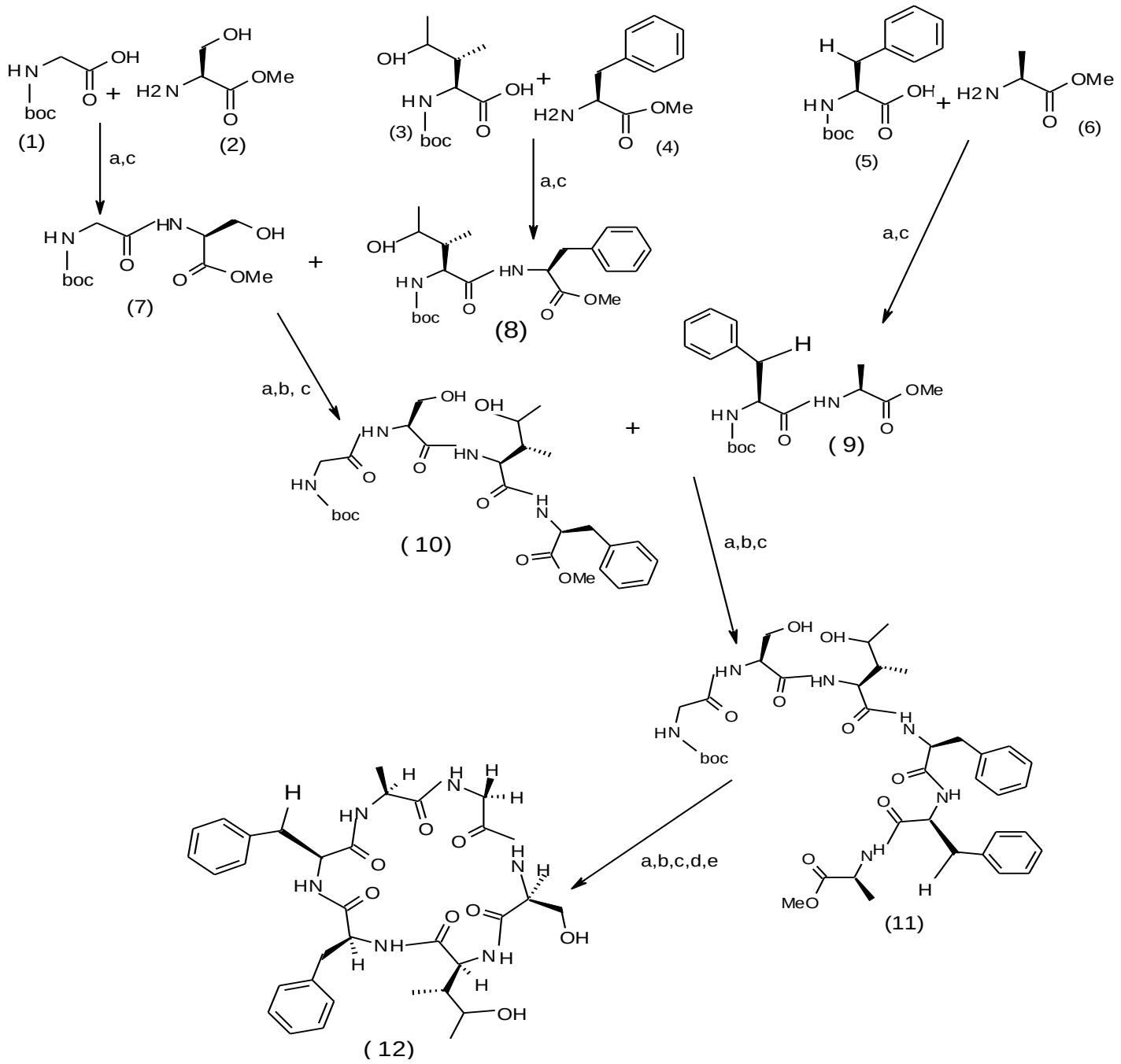
The cyclization of linear octapeptide was done by using p-nitrophenyl ester method (Bodanszky, 1984). The ester group of linear fragment was detached by using LiOH and the p-nitrophenyl ester group was introduced. In order to introduce p-nitrophenyl ester group, the Boc-peptide carboxylic acid (1.5 mmol) was added to chloroform (15 ml) at 0°C, to which p-nitrophenol (0.27 g, 2 mmol) was added, and allowed to stir for 12 h at RT. The reaction mixture was filtered and filtrate was washed by using $NaHCO_3$ solution (10%) until excess of p-nitrophenol was removed. The filtrate was finally washed with 5% HCl (5 ml) to obtain Boc-peptide-pnp ester. To the already mentioned Boc-peptide-pnp-ester (1.2 mmol) in $CHCl_3$ (15 ml), CF_3COOH (0.274 g, 2.4 mmol) was added and allowed to stir for 1 h at room temperature. The reaction mixture was washed with 10% $NaHCO_3$ and the organic layer was dried over anhydrous Na_2SO_4. To the Boc-deprotected peptide-pnp-ester in $CHCl_3$ (15 ml), N-methyl morpholine (1.4 ml, 2 mmol) was added and kept at 0°C for 7 days. The reaction mixture was washed with 10% $NaHCO_3$ till the byproduct p-nitrophenol was removed completely.

Cytotoxic activity

Preliminary cytotoxic activity by brine shrimp lethality assay (BSLA)

Brine shrimp eggs were obtained from the aquarium shop, Nashik. Artificial sea water was prepared from (1% NaCl) prepared by using nitrate, phosphate, and silicate-free sea-salt and distilled water (35 g/L) at 25°C under constant illumination. The salt water solution was aerated continuously during incubation with an aquarium air pump.

The seawater was placed in a small plastic container (hatching chamber) with a partition for dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber while the lamp above the other side (light) will attract the hatched shrimp. Two days were allowed for the shrimp to hatch and mature as nauplii



Scheme 1. Synthetic route for Delavayin A. Where: a = DIPC, NMM, CHCl3, RT, 24h, b = TFA, NMM, RT, 1 h, C = LiOH, THF:H_2O(1:1), reflux, 15 min, d = pnp-, CHCl3, RT, 12 h, e = NMM, $CHCl_3$, 0°C, 7 days.

(larva). After two days, when the shrimp larvae are ready, 4 ml of the artificial seawater was added to each test tube containing different concentration of drug and 10 brine shrimps were introduced into each tube.

Thus, there were a total of 30 shrimps per dilution. Then the volume was adjusted with artificial seawater up to 5 ml per test tube. The test tubes were left uncovered under the lamp. The number of surviving shrimps were counted and recorded after 24 h. Using probit analysis, the lethality concentration (LC_{50}) was assessed at 95% confidence intervals. The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the activity of the compound (Bussmann et al., 2011; Tagne et al., 2014). The

Table 1. Results for Cytotoxic activity by using brine shrimp assay.

Compound	Conc. (ppm or µg/ml)	Number of surviving Nauplii after 24 h			Total number of survivors	% Mortality
		T1	T2	T3		
Dela A	1000	4	4	5	13	56.66
	500	5	4	5	14	53.33
	250	6	6	7	19	36.66
	125	7	6	7	20	33.33
	62.5	7	8	8	23	23.33
	31.25	8	9	9	26	13.33
	0	10	10	10	30	0

*T=Trial, LC_{50}=460.9 µg/ml.

Table 2. Data of cytotoxic activity against human tumor cell lines.

Compound	% Mortality at various concentration						LC 50 (µg/ml)
Conc.(µg/ml)	25	50	100	150	200	250	
Dela A	16.66	30	43.33	63.33	80	90	117.5

results of activity are shown in Table 1.

In vitro cytotoxic activity against Human tumor cell lines

The cytotoxic activity against human tumor cell lines PC3 and HL 60 was carried out at ACTREC Mumbai, by following standard protocol (www.actrec.gov.in). The activity was carried out by sulphorhodamine B assay (Vichai, 2006; Skehn, 1990). The cell lines were grown in RPMI 1640 medium containing 2 mM L-glutamine and 10% fetal bovine serum. For screening, cells were inoculated into 96 well microtiter plates in 100 µl, followed by incubation at 37°C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to the addition of experimental drugs. After 24 h, one 96 well plate containing 5 × 103 cells/well was fixed *in situ* with TCA to represent a measurement of the cell population (Tz) at the time of addition of drug. Experimental drugs were initially added to DMSO at 100 mg/ml and diluted to 1 mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to 100, 200, 400 and 800 µg/ml with complete medium. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations, that is 10, 20, 40 and 80 µg/ml. Plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded; plates were washed and air dried. SRB solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing with 1% acetic acid and air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth has been calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100. Six absorbance measurements [at time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)] were used to calculate the percentage growth inhibition. Percentage growth inhibition at each of the drug concentration was calculated as:

[(Ti–Tz)/(C–Tz)] × 100 for concentrations for which Ti ≥ Tz (Ti–Tz) positive or zero
[(Ti–Tz)/Tz] × 100 for concentrations for which Ti < Tz. (Ti–Tz) negative

Adriamycin was used as a standard for carrying out the activity. The data of cytotoxic activity against different human tumor cell lines is shown in Table 2. The results of activity are plotted as graph of drug conc. vs % control growth and are shown in Figures 1 and 2. The activity of standard drug Adriamycin and synthesized compound against human tumor cell lines are shown in Figures 3 and 4.

Antimicrobial assay

Antimicrobial assay for synthesized cyclic heptapeptide was carried out by using tube dilution technique (Bauer, 1996; Shinde, 2008, 2010). The bacterial strains and fungal strains were obtained from the National collection of industrial micro-organisms (NCIM), branch of National chemical laboratory (NCL) Pune, India. The antibacterial activity was performed against four bacterial species *B. subtilis* ATCC NO 6051, *S. aureus* ATCC NO 25923, *E. coli* ATCC NO 25922 and *P. aeruginosa* ATCC NO 9721) and antifungal activity was performed against *Candida albicans* ATCC NO 2091 and *Aspargillus niger* ATCC NO 10594. For carrying out antibacterial and antifungal activity, Benzyl penicillin and Fluconazole were used as standards. A solution of the compound was prepared in DMF and a series of doubling dilutions prepared with sterile pipettes so

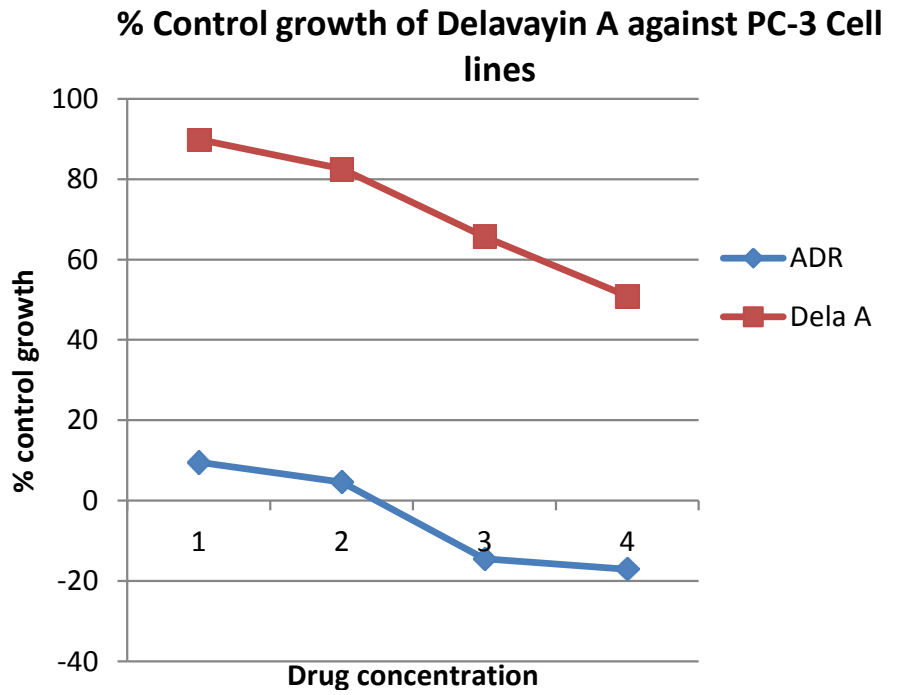


Figure 1. Cytotoxic activity of Delavayin A against PC-3 cell lines at different concentration in comparision with Adriamycin.

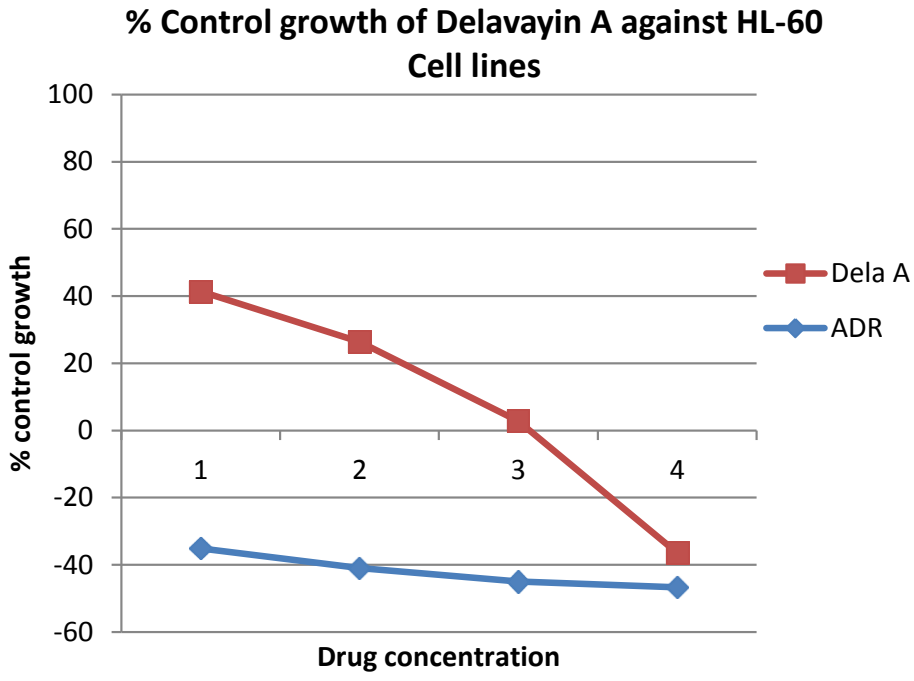


Figure 2. Cytotoxic activity of Delavayin A against HL-60 cell lines at different concentration in comparision with Adriamycin.

as to make concentrations as 100, 50, 25, 12.50, 6.25, 3.125, 1.562 and 0.781 µg/ml, respectively. A standard volume of nutrient broth medium was added to each of a series of sterile stoppered test tubes and a control tube containing no antimicrobial agent was also

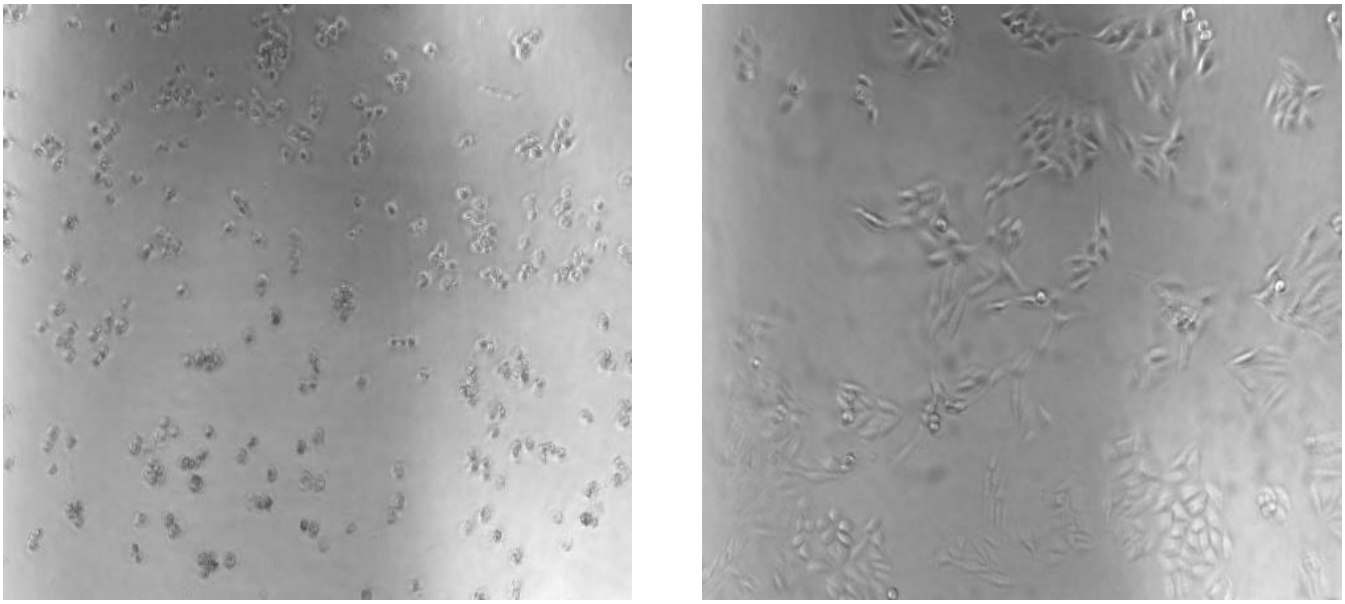


Figure 3. Cytotoxic activity of Adriamycin against PC3 cell lines (a) and HL-60 cell lines (b).

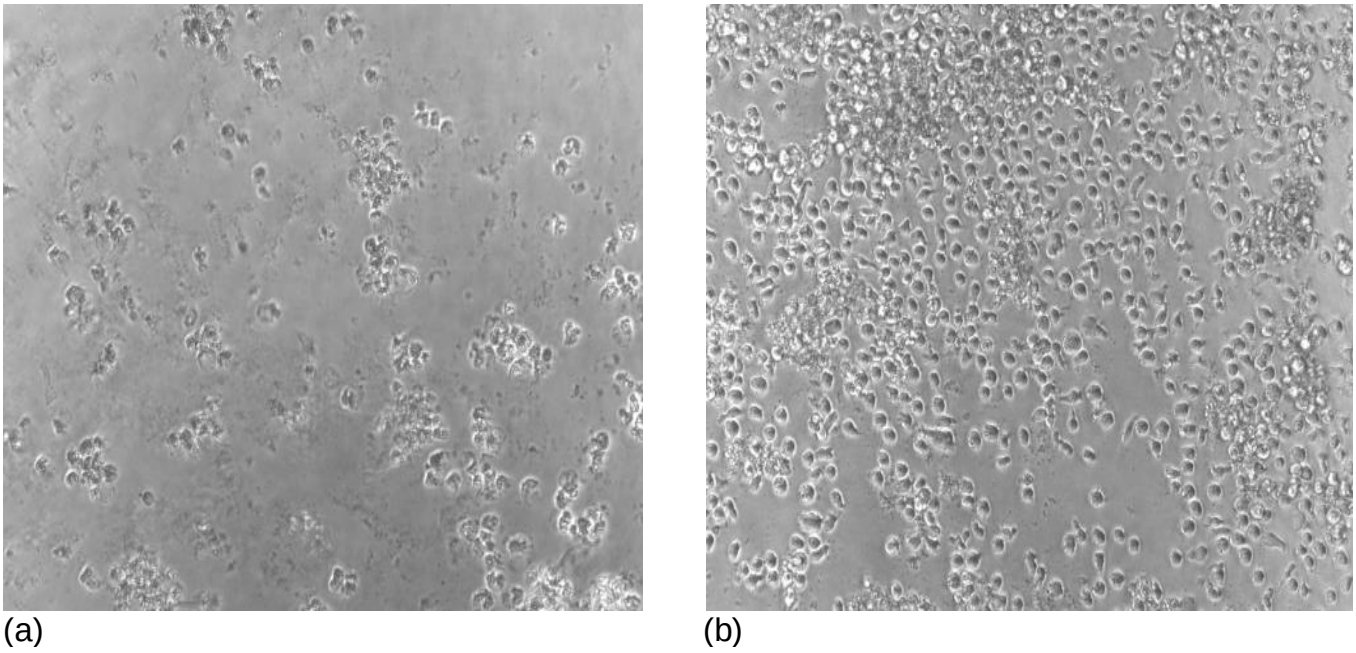


Figure 4. Cytotoxic activity of delavayin A against PC3 cell lines (a) and HL-60 cell lines (b).

included. The inoculum consisting of overnight broth culture of micro-organisms was added to separate tubes. The tubes were incubated at 37°C for 24 h and examined for turbidity. The tube with highest dilution showing no turbidity was the one containing compound with MIC. From the screening data of antibacterial and antifungal activity revealed that the synthetic peptide is found to be active. The results are shown in Table 3.

RESULTS AND DISCUSSION

Spectral data

- Physical state: Semisolid mass
- IR data: 3316.3(NH-stretch.), 2932.2(aliph.-CH stretch.),

Table 3. Minimum inhibitory concentration for antimicrobial activity.

Compound	Percent control growth (Average value, n=3)			
Conc. (µg/ml)	**10**	**20**	**40**	**80**
PC3 (against sample)	89.9	82.6	65.7	50.9
PC3 (against standard)	9.5	4.6	-14.2	-17.1
HL 60 cell lines (against sample)	76.3	67.12	47.5	10.1
HL 60 cell lines (against Standard)	-35.1	-41.0	-45.0	-46.7

2854.9, 1648.5(C=O of amide), 1534.7(-NH bend), 1453.1 (-CH bend).
- FABMS: showed M^+ ion peak at *m/z* 640.3
- ^{13}C NMR: showed six amide carbonyl carbon (δ170.01, 171.00, 171.16, 171.56, 172.65, 173.60).
- δ 43.86 for α Gly, δ 170.08 for C=O of Gly, δ 55.45 for α position of Serine, δ 63.12 for β position of Serine, δ 60.39, 40.01, 15.11,21.09 for α,β,γ and δ positions respectively of α hydroxy isoleucine, δ 56.24 , 39.91, for α and β positions of Phe, δ 171.11 for C=O of Phe, 137.43 for γ position of Phe, δ 52.11 for α position of Ala, δ 16.57 for β position of Ala, δ 173.11 for C=O of Ala.
- ^1H NMR: showed six amide protons (δ 8.20, 8.70, 9.15, 9.25, 9.33, 9.46, 10.18).
- δ 4.90 (HN of Gly), δ 4.16(dd, Hα of Gly), δ 10.20 (dd, C=O of Gly), δ 5.25 (dd, Hα of ser), δ 4.40 (HN of Ser), δ 9.47 (d, C=O of Ser), δ 1.01 (3H,d,7.1)γ CH_3 of γ hydroxy Ile, δ 3.85(1H,dd,6.0,12.3) for γ hydroxy Ile, δ 5.32(1H,dd,3.9, 8.5, 11.1)α Phe, δ 7.39(2H,d,7.2) δ Phe, δ 4.69 (1H,dd,6.2,12.3) α Phe, δ 3.44 (2H,dd,6.3) β Phe, δ 4.48 (1H,d,6.6, 6.9) α Ala, δ 4.53(1H,d, 3.9, 6.9) for α Ala, δ 1.53(3H,d, 6.9) for β Ala, δ 9.31(1H,D, 3.6) N-H of Ala.
- Elemental analysis: C: 60.9 (60.17), H: 6.54 (6.63), N: 12.12 (13.16).

DISCUSSION

The compound was synthesized with good yield by using solution phase technique. Anti-cancer activity of synthesized compound was carried out by SRB assay and had shown to possess moderate activity against PC 3 and HL-60 cancer cell lines when compared against adriamycin standard. The synthetic peptide has shown prominent activity against *Bacillus subtilis* and *Staphylococcus aureus* (Gram positive bacteria) and less activity against *Escherichia coli* and *Pseudomonas aeruginosa* (Gram negative bacteria) in comparison with standard drug benzyl penicillin. The synthesized molecule has also shown moderate activity against fungal strains *Candida albicans* and *Aspargillus niger*. As N-methylated analogs of most of cyclic peptides are found to show increase in the activity, there is scope for synthesizing analogs of lead molecule for the development of potent cytotoxic and antimicrobial agent.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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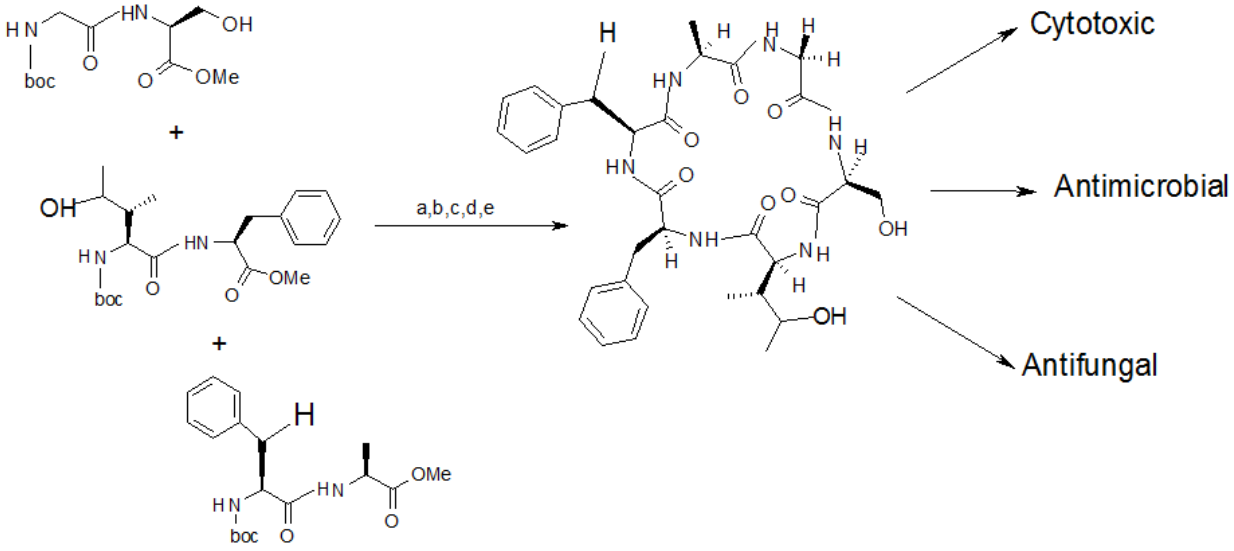
Appendix 1. Graphical abstract.



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