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Full Length Research Paper

Malva neglecta: A natural inhibitor of bacterial growth and biofilm formation

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***Malva neglecta*, a wild plant that grows in different parts of Lebanon, was noted by residents to have soothing effects if taken during episodes of respiratory tract infections. This study was designed to test for the ability of this plant to inhibit bacterial growth and biofilm formation of clinical bacterial isolates. The results showed that while the aqueous extract of the leaves of the plant did not show any antibacterial effect on the tested bacterial isolates, the methanol extract clearly demonstrated an ability to inhibit the growth of the isolates tested. The agar dilution method revealed that the lower concentrations of the methanol extract of *M. neglecta* inhibited some isolates, but the inhibition was noted to increase with an increase in the concentration of the extract until at a ratio of 0.3 (volume of extract to volume of the agar medium), the growth of all the tested organisms was completely inhibited. The methanol extract of the plant was also capable of inhibiting the formation of biofilms by many of the clinical isolates tested. The active component in the *M. neglecta* if identified, purified and proved safe for human consumption, may prove to be a new effective antibacterial agent.**

Key words: Antibacterial agents, ethnobotany, biofilms, *Malva neglecta*, medicinal plants, plant extracts.

INTRODUCTION

The advent of antimicrobial agents was probably the most important achievement in modern medicine. The lives of millions were saved as these were put in use to treat very acute and difficult infections like those following surgery and organ transplantation or those affecting preterm babies or the elderly. Some antimicrobial agents were even effective as cancer chemotherapeutic agents. However, these agents were abused and resulted in the emergence of drug resistant, multidrug resistant and exceptionally drug resistant organisms (Alanis, 2005; Kapil, 2005). Many infections started to become very hard to treat due to the spread of these aggressive pathogens. Thus, antimicrobial resistance, in addition to increasing the mortality rate of patient, increased healthcare expenses and threatened the achievement of the development goals even in advanced societies. In short, antimicrobial resistance, nowadays, is probably the most serious threat to global health.

What magnified the problem more were the reports that bacterial biofilms played a major role in initiating more than three quarters of microbial infections in the human body (O'Toole et al., 2000), including otitis media,

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osteomyelitis, native valve endocarditis, cystic fibrosis pneumonia as well as urinary tract infections (Costerton et al., 1999). A biofilm, which is a buildup of microorganisms in mainly, a polysaccharide environment (O'Toole et al., 2000; Sutherland, 2001), was shown to be involved in the change of organisms from a planktonic to a sessile mode of growth. Accordingly, the biofilm forming organisms were protected from any harsh surrounding conditions (Costerton et al., 1999), including exposure to antimicrobial agents. Mature biofilms (> 7 days) were demonstrated to be notably resistant to 500 to 5,000 times the concentrations of these agents than were necessary to kill free floating (planktonic) cells of the same organism (Khoury et al., 1992). Common biofilm formers are numerous and include *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes, Klebsiella pneumonia, Proteus mirabilis* and *Pseudomonas aeruginosa* (Donlan, 2001).

Finding new methods to combat these organisms including searching for new antimicrobial drugs including antibiotics has been a major concern to all public health authorities. Ethno-pharmacologists, botanists, microbiologists and natural-product chemists, have not seize to work on plants to find new phytochemicals that may serve as a solution for this wide spread and still magnifying threat (Ncube et al., 2008; Laxminarayan et al., 2013; WHO, 2016).

Traditional and even modern medicine depends fully or in part on medicinal plants that were and still are a very important bio-resource of drugs. Historically, many of these plants were used by regular people over time and were recognized for their positive effect (Neube et al., 2008). As reports indicated that the organic solvent extracts of both the leaves and flowers of other species of the same genus (*Malva sylvestris* and *Malva moschata),* had high bactericidal activity, anti-ulcerogenic and anti-inflammatory effects in patients when used for respiratory tract, GI tract and skin infections (Kumarasamy et al., 2002; Çakılcıoğlu et al., 2010; Razav et al., 2011; Mohammad Eini et al., 2014; Mirghiasi et al. 2015), the present study was performed using the plant, *Malva neglecta,* commonly used, in the Northern Bekaa region and other rural regions of Lebanon, as a medicinal plant for treatment of respiratory tract infection

M. neglecta is a wild plant that is also known as the dwarf mallow or common mallow and belongs to the mallow family – *Malvaceae*. Its flowers are regular (actinomorphic), 1.5-2.5 cm wide with 5 petals that are white, pinkish or red-veined with notched tips. The leaves of *M. neglecta* are alternate, long-stalked, stipulate and blade-kidney shaped (Nature Gate, 2017).

M. neglecta was also reported to have many additional benefits. It demonstrated anti-ulcerogenic and anti-inflammatory activities in patients with osteoarthritis, and was used as an expectorant, laxative and for the maturation of abscesses, stomach ache, menstrual disorders, abdominal pain, sore throat, vaginitis and constipation (Çakılcıoğlu et al., 2010; Mirghiasi et al., 2015).

This study aimed at determining whether the leaves of *M. neglecta,* had antibacterial effects and/or had the ability to inhibit the formation of biofilms by different pathogenic bacteria.

MATERIALS AND METHODS

Plant used

Fresh samples of the leaves of the plant, *M. neglecta,* which grows in the wild, were obtained from the Deir El Ahmar region of the Governorate of Bekaa in Eastern Lebanon, identified by our reference botanist and then immediately sent to the microbiology laboratory for processing.

Bacterial isolates

The bacterial isolates used in the study were clinical isolates courteously provided by the Clinical Microbiology Laboratory of the Lebanese American University Medical Center- Rizk Hospital (LAUMC- RH). Specifically, the isolates used were the following: 3 isolates of *Pseudomonas aeruginosa* (designated in the study as isolates 1, 2 and 3) and one isolate of each of the following organisms: *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus aureus* and *Streptococcus pyogenes.* The identity of the isolates was confirmed by Gram staining and standard tests (Jorgensen and Pfaller, 2015) were performed. For the definitive identification of the Gram negative bacilli, API 20E kits ((Biomerieux-France) were used.

Aqueous extraction and testing for anti-bacterial activity

Preparation of the aqueous extract

The fresh *M. neglecta* leaves were boiled in water until the liquid became thick. A portion of the extract was autoclaved while another was used without autoclaving.

Testing for anti-bacterial activity of the aqueous extract

The antibacterial effect of the aqueous extracts was performed by using the disc agar diffusion and the well agar diffusion methods. The recommended Mueller-Hinton agar (MHA) was prepared and used in both methods as recommended (CLSI, 2014).

The disc agar diffusion method: The MHA plates were seeded with a 0.5 McFarland equivalent turbidity of the fresh test organisms (*S. aureus*, *E. coli* and *P. aeruginosa* isolate 1). The discs used were filter paper discs soaked with the aqueous extract (20 µl of the extract were added to each disc) and were inserted on the surface of the agar as recommended (CLSI, 2014). The plates were incubated at 35°C for 24 h after which the appearance of zones of inhibition around the discs were noted and measured (when present). The test for each organism was done using the autoclaved and the unautoclaved aqueous extract.

The well agar diffusion method: The MHA plates were seeded with a 0.5 McFarland equivalent turbidity of the fresh test organisms (*S. aureus, E. coli* and *P. aeruginosa* isolate 1). Using a cup borer, an 8.5 mm well in the middle of the plate was filled with 100 µl of

the extract (Perez et al., 1990). The plates were then incubated at 35°C for 24 h after which the appearance of zones of inhibition around the wells were noted and measured (when present). The test for each organism was done using the autoclaved and the unautoclaved aqueous extract.

Methanol extraction and testing for anti-bacterial activity

Preparation of the methanol extract

The fresh *M. neglecta* leaves were chopped and mixed with 80% methanol in a blender. The extract was then transferred to an Erlenmeyer flask and kept in an orbital shaker for 1 week, after which the extract was filtered using vacuum filtration and was ready for use.

Testing for anti-bacterial activity of the methanol extract

The antibacterial activity of the methanol extract was tested using the agar dilution method (Ncube et al., 2008). Trypticase soy agar (TSA) plates were prepared and autoclaved as recommended by the manufacturer. A portion of the agar was poured into the first half of a bi-Petri dish (to be used as control) in each plate that will be used in the experiment. The Erlenmeyer flask containing the remaining melted TSA agar was then put in a water bath at 70°C, and a volume of *M. neglecta* methanol extract was added to the agar to obtain a ratio of 0.14 of the methanol extract volume to the volume of the TSA agar. The procedure was then repeated twice to prepare TSA agar media containing a ratio of methanol extract volume to that of TSA agar of 0.16 and 0.30, respectively. The melted agars were kept in the water bath (at 70°C) for 15 min to allow for the evaporation of the methanol, after which each of the 3 melted agars were poured in the second half of each of the bi-petri dishes that were used in the testing of the antibacterial effect of each of the different organisms used in the study.

After the turbidity of each of the tested organisms (*P. aeruginosa*, isolates 1, 2 and 3, *E. coli, K. pneumoniae, P. mirabilis, S. aureus* and *S. pyogenes*) was adjusted to that of a 0.5 McFarland standard (in saline), the prepared bi-plates were each seeded by the test organisms (individually) using different sterile swabs, for each part of each plate, incubated at 35°C for 24 h and then checked for the growth of the organisms on the surface.

Effect of the methanol extract on biofilm formation

Preparation of the bacterial isolates

From fresh agar plates, each of the test organisms was used to inoculate a 10 ml trypticase soy broth (TSB) tube with 1% glucose. The inoculated TSB tubes were left in the incubator at 37°C for 24 h after which, the culture tubes were diluted 100 times with fresh media.

Effect of the extracts on biofilm formation

To test for the formation of biofilms by the isolates and possible inhibition of the process by the methanol extract of *M. neglecta*, a method very slightly modified from that used by Mathur et al. (2006) was used. The methanol extract was added to the test wells of the 96 well flat-bottom tissue culture plates and the plates were left to dry in the incubator under aseptic conditions. Upon drying, 200 µl of sterile TSB were added to the wells of the plates with 10 µl of the diluted cultures (previous section) and incubated at 35°C for 24 h. The contents of the wells were then gently discarded by repeated soft tapping, after which the wells were washed with phosphate buffered saline (PBS, pH of 7.2) several times. Then, 0.2% sodium acetate was added to fix any biofilms that may have formed and a 0.1% solution of crystal violet was finally added to stain the biofilms, when present. Excess stain was then removed with deionized water and the plates were left to dry. The optical densities were later determined by using a microplate auto-reader at 570 nm wavelength. To have a precise result, each of the test samples (and controls) was performed in 16 wells. The reported optical densities in the study were the averages of the 16 readings of each sample.

RESULTS

Antibacterial effect of the aqueous extract

Using both the disc agar diffusion and well agar diffusion methods, the aqueous extract of *M. neglecta* did not demonstrate any antibacterial effect. No zones of inhibition of growth were detected in any of the test plates for any of the 3 bacterial isolates used: *E. coli, P. aeruginosa* (isolate 1) and *S. aureus*.

Antibacterial effect of the methanol extract

Unlike the aqueous extract, the methanol extract showed obvious antibacterial characteristics. At a ratio of extract volume to agar volume of 0.14, the extract of *M. neglecta* inhibited the growth of the *P. aeruginosa* (isolate 2) and *S. pyogenes*, but had no effect on the other isolates (Table 1).

When the ratio of extract volume to agar volume was slightly increased to 0.16, the growth of *S. pyogenes S. aureus* and *K. pneumoniae* was inhibited totally, while the growth of *P. aeruginosa* (isolate 1) was partially inhibited. It was noted, however, that the *P. aeruginosa* (isolate 2) which was totally inhibited at the ratio of 0.14 (v/v) grew moderately at the slightly higher ratio of 0.16 (v/v) (Table 2). Upon raising the ratio of extract volume to agar volume to 0.3, the growth of all the tested clinical isolates was totally inhibited (Table 3).

Effect of the extracts on biofilm formation

Table 4 represents the optical densities read for each of the samples tested and controls. The same results are represented graphically in Figure 1. The results clearly indicate the ability of all the chosen clinical isolates to form biofilms on the bottom of the tissue culture plates. Whereas it was clear that the *M. neglecta* methanol extract was not capable of inhibiting the formation of biofilms by: *P. aeruginosa* (isolate 3), *E. coli, K. pneumoniae* and *S. pyogenes*, it was clearly evident that it was efficient in inhibiting biofilm formation by two of the *P. aeruginosa* isolates (1 and 2), *P. mirabilis* isolate and the *S. aureus* isolate (Figure 1).

Table 1. Effect of the methanol extract of *M. neglecta* on the growth of the bacterial isolates, at a ratio of extract volume to agar volume of 0.14. -: No growth; +++: confluent growth.

Bacterial	Ratio of 0.14	
	Control	Experiment
Escherichia coli	+++	+++
Pseudomonas aeruginosa (isolate 1)	+++	+++
Pseudomonas aeruginosa (isolate 2)	+++	-
Pseudomonas aeruginosa (isolate 3)	+++	+++
Staphylococcus aureus	+++	+++
Streptococcus pyogenes	+++	-
Proteus mirabilis	+++	+++
Klebsiella pneumoniae	+++	+++

Table 2. The effect of the methanol extract of *M. neglecta* on the growth of the bacterial isolates, at a ratio of extract volume to agar volume of 0.16. -: No growth; +: weak growth; ++: moderate growth; +++: confluent growth.

Bacterial	Ratio of 0.16	
	Control	Experiment
Escherichia coli	+++	+++
Pseudomonas aeruginosa (isolate 1)	+++	+
Pseudomonas aeruginosa (isolate 2)	+++	++
Pseudomonas aeruginosa (isolate 3)	+++	+++
Staphylococcus aureus	+++	-
Streptococcus pyogenes	+++	-
Proteus mirabilis	+++	+++
Klebsiella pneumoniae	+++	-

Table 3. The effect of methanol extract of *M. neglecta* on the growth of the bacterial isolates, at a ratio of extract volume to agar volume of 0.30. -: No growth; +: weak growth; ++: moderate growth; +++: confluent growth.

Bacterial	Ratio of 0.30	
	Control	Experiment
Escherichia coli	+++	-
Pseudomonas aeruginosa (isolate 1)	+++	-
Pseudomonas aeruginosa (isolate 2)	+++	-
Pseudomonas aeruginosa (isolate 3)	+++	-
Staphylococcus aureus	+++	-
Streptococcus pyogenes	+++	-
Proteus mirabilis	+++	-
Klebsiella pneumoniae	+++	-

DISCUSSION

The inability of the aqueous extract of *M. neglecta* to show any effect on the growth of the 3 bacterial isolates tested (*E. coli, P. aeruginosa* (isolate 1) and *S. aureus*), may have indicated that the inhibitory bioactive compound may have been thermolabile and was destroyed during the boiling process. However, traditional healers depended primarily on aqueous extracts of the plant, although organic extracts were found to yield more

Table 4. Average optical density (O.D.) readings at a wavelength of 570 nm, of the different cultures tested in this study. Pa: *Pseudomonas aeruginosa* (isolates 1, 2 and 3); Ec: *E. coli;* Pm: *P. mirabilis;* Kp: *K. pneumoniae;* Sa: *S. aureus;* Sp: *S. pyogenes*; E: Methanol extract of *M. neglecta*; C: Control.

Isolate	Pa 1 + E	Pa 1	Pa 2 + E	Pa 2	Pa 3 + E	Pa3	Ec + E	Ec
O.D.	0.650004	0.718142	0.84054	1.094683	0.610321	0.591927	0.485839	0.50045
Isolate	Kp + E	Kp	Sa + E	Sa	Sp + E	Sp	Pm + E	Pm
O.D.	0.646881	0.560772	0.440994	0.485946	0.77043	0.377233	0.454117	0.567701
	E	C						
O.D.	0.400989	0.331868						

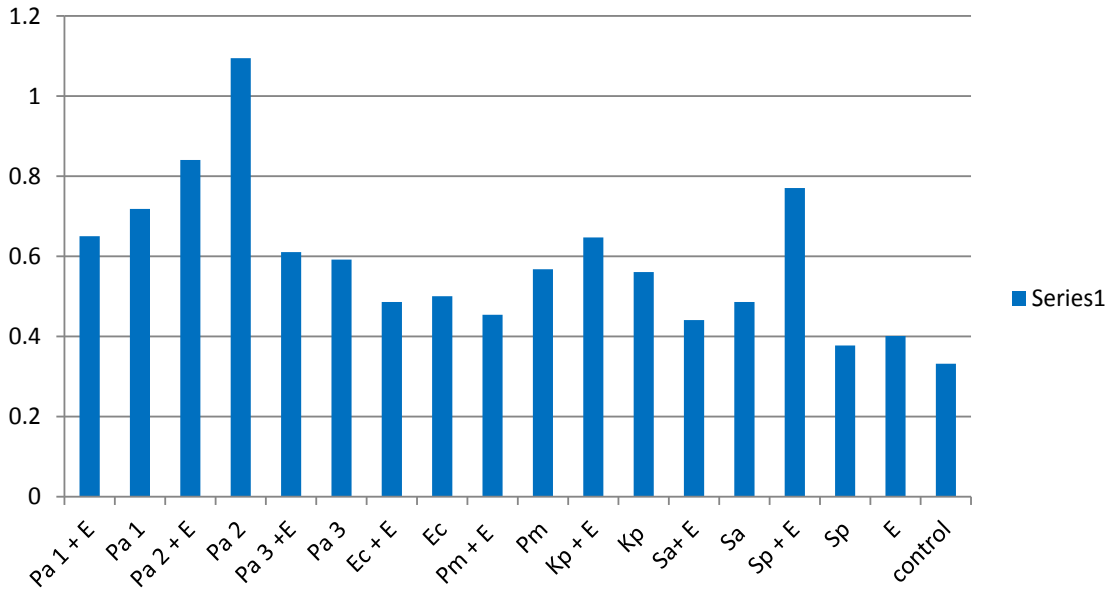

Figure 1. Graphic representation of the data in Table 4. X axis: Samples tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm; Pa: *P. aeruginosa (isolates 1, 2 and 3);* Ec: *E. coli;* Pm: *P. mirabilis;* Kp: *K. pneumoniae;* Sa: *S. aureus;* Sp: *S. pyogenes;* C: Control.

consistent results regarding antimicrobial activity (Parekh et al., 2005). The result may have been caused, however, by what was noted by Silva et al. (2005), that the disk agar diffusion assay can lead to unreliable results in case mixtures containing different constituents, because of different diffusion rates. Another reason may have been not soaking the disks with the aqueous extract for hours as as suggested by Mbata et al. (2006).

A previous report by Parekh et al. (2006) noted that most of the identified antimicrobial chemicals from plants were water insoluble, a reason why extracts by organic solvents were more effective. Moreover, Imtiaz et al. (2012) found that the *M. neglecta* organic solvent extract was much more effective in inhibiting the growth of *E. coli, K. pneumoniae, Salmonella* Typhi and *B. subtilis* than the aqueous extract of the plant. Taking the results of these studies into consideration, methanol was selected and was a very appropriate organic solvent for this study.

The methanol extract proved to have a very notable effect on the growth of the bacterial isolates tested. This effect increased with the increase in the volume of the methanol extract added to the melted agar. Whereas, there was complete inhibition of only two isolates at a ratio of 0.14 (Table 1), there was complete inhibition of 3 isolates and partial inhibition of 2 others when the ratio was increased to 0.16 (Table 2). At a ratio of 0.3, the methanol extract completely inhibited the growth of all the clinical bacterial isolates used in this study (Table 3).

Although, at higher concentrations of the extract, the inhibition of growth was clear, yet the effect obviously started at lower concentrations. The growth of bacteria at lower concentrations does not indicate the absence of an effect as it has been verified previously (Zhanel et al., 1992) that the sub-inhibitory antimicrobial concentrations may have produced numerous effects including altering

bacterial morphology and growth, affecting bacterial virulence factors and altering bacterial susceptibility to host immune defenses. However, further studies are needed to discover whether this happened in our case or if the extract exerted any effect at the molecular levels.

The use of the agar dilution method in this study proved to be more suitable for our purposes than either the agar diffusion or well diffusion methods. Mathur et al. (2006) and the European Committee for Antimicrobial susceptibility testing – EUCAST (2003) recommended the use of Muller-Hinton agar for similar tests, some researchers reported the use of nutrient agar (Meyer and Afolayan, 1995; Grierson and Afolayan, 1999). TSA was chosen for use in this study as it proved to be very suitable for the growth of the test isolates, with an inoculum density equivalent to the 0.5 McFarland standard as recommended in the standard procedure (Costerton et al., 1999; CLSI, 2014).

Antibacterial assays showed that the methanol extracts of both the leaves and flowers of other species of the same genus, *M. sylvestris* had inhibitory activity against human pathogens like*, E. coli, Listeria monocytogenes* and *Streptococcus agalactiae.* A comparison between the antibacterial effects of the aerial and the root organs of *M. sylvestris* showed that the main effect belonged to the aerial parts and very strongly against *Pasteurella multocida* (Eini et al., 2014). Also, the methanol extract of the seeds of *M. moschata* showed antibacterial activity against certain bacteria (Kumarasamy et al., 2002).

The antibacterial effect that was demonstrated in the methanol extract of *M. neglecta* and not in the aqueous extract of the plant in this study, was also reported by Jasim (2006), who found out that the aqueous extract of *M. neglecta* did not have antibacterial activity against *S. aureus, S. pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis*, but the petroleum ether extract of the same plant inhibited the growth of these organisms.

This study is believed to be the first to demonstrate the ability of *M. neglecta* methanol extract to also inhibit biofilm formation of several clinically important bacterial strains (Table 4 and Figure 1). This finding is extremely important as one of the most challenging problems in antibacterial therapy remains difficult in targeting bacteria in already formed biofilms (Khoury et al., 1992).

Conclusion

M. neglecta, being a plant that grows in the wild, seems to be a powerful medicinal plant as it proved to have the ability to inhibit the growth and biofilm formation of many clinically significant bacteria and may be a very promising source of new antibacterial medications. Further work is still needed, however, to determine, as recommended for previous similar studies (Iwu et al., 1999), the active compounds in the plant, purify them, and evaluate if they can be used as new medications that do not cause any harm to the host or the normal microbiota.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparative effect of *Nauclea latifolia* leaf fractions on blood glucose and lipid profile parameters of alloxan induced-diabetic rats

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The antidiabetic effects of methanol, N-hexane and ethyl acetate leaf fractions of *Nauclea latifolia* were investigated in diabetic model rats. 150 and 300mg/kg fractions of *N. latifolia* were administered orally to the experimental animals at two weeks interval, while their blood glucose levels were taken daily. At the end of the experiment, their lipid profiles were assayed using standard methods. The research data indicated significant decreases (P=0.05) of the blood glucose in all the fractions at a dose-dependent manner (ethyl acetate (150 mg/kg = 47.83%; 300 mg/kg = 64.17%), N-hexane (150 mg/kg = 58.45%; 300 mg/kg = 64.18%), methanol (150 mg/kg = 44.5%), except for the 300 mg/kg dose methanol fraction (0.82%) as compared to the increased level in the negative control (33.33%). In the lipid profile assay, there were also a dose dependent significant decrease (P=0.05) of serum Total Cholesterol (TC) and Low Density Lipoprotein (LDL) cholesterol in all the fraction groups (ethylacetate (150 mg/kg- TC = 69.88 ± 8.52 mg/dl, LDL-cholesterol = 8.23±7.76 mg/dl; 300 mg/kg-TC = 51.08±9.93 mg/dl, LDL-cholesterol = 6.44±7.66 mg/dl), N-hexane (150 mg/kg-TC = 73.16 ± 18.62mg/dl, LDL-cholesterol = 33.24±16.19 mg/dl; 300 mg/kg-TC = 69.78±8.41 mg/dl, LDL-cholesterol = 9.29±5.62 mg/dl and methanol (150 mg/kg; TC = 116.86 ± 13.34 mg/dl, LDL-cholesterol = 50.68±14.13 mg; 300 mg/kg-TC = 108.66 ± 12.77 mg/dl, LDL-cholesterol = 42.09±9.93mg/dl) as compared to the high concentration of the negative control group (TC = 383.76 ± 79.68 mg/dl, LDL-cholesterol = 299.46 ± 79.23 mg/dl). Ethylacetate and N-hexane fractions showed significant reductions (P=0.05) of TC and LDL-cholesterol as compared to the positive control (TC=116.36±14.69 mg/dl, LDL-cholesterol = 32.06±13.23mg/dl) also at a dose-dependent manner; thus, portraying a more efficient hypolipidaemic activity than the standard drug. This antidiabetic research on *N. latifolia* suggest that ethylacetate fraction produces the best effect, followed by N-hexane and lastly by methanol fraction.

Key words: *Nauclea latifolia,* ethyl acetate, n-hexane, methanol, leaf fractions, glibenclamide, antidiabetic, blood glucose, lipid profile.

INTRODUCTION

Diabetes mellitus can be defined as an increase in blood glucose without the presence or non-enough pancreatic insulin secretion which could involve the concurrent impairment of insulin action (Martha, 2009). It is an endocrine disorder characterized by aberration in carbohydrate, protein, blood relating functions and fat

metabolism as a result of complete or relative insufficiency of insulin secretion and action (American Diabetes, 2006; Canadian Diabetes Associates, 2008).

Herbal medicine entails the usage of herbs and plant parts (roots, stems, barks, or even fruits) in enhancing an improved health (Bussmann and Sharon, 2006). The recommendation by WHO on diabetes mellitus has shown the importance of research on hypoglycemic agents from medicinal plants. The photochemistry and their pharmacological values shows that about 800 plants could be applied for the treatment of diabetes traditionally throughout the world (Kavishankar et al., 2011).

Nauclea latifolia Smith, (Rubiaceae) is known commonly as Pin cushion tree and a strangling shrub that is native to tropical Africa and Asia. *N. latifolia* is largely used in the traditional folklore (Akpanabiatu et al., 2005). The pharmacological usage of *N. latfolia* leaves reported include hypoglycemic, hypolipidemic and hypo-cholesterolemic property (Gidado et al., 2008; Asanga et al., 2013), antihypertensive property (Nworgu et al., 2008).

Blood sugar level could be measured using a glucose meter either in mg/dl or mmol/L of blood. The normal value of glucose level for an average person is 4.5 to 7.0 mmol/L (81 to 126 mg/dl) (Briscoe, 2006). Values greater than 13 to 15 mmol/L (230 to 270 mg/dl) are considered high; usually referred to as hyperglycaemic needs to be closely monitored to return to normalcy. The patient required an urgent medical attention if after 2-3 tests blood sugar levels continue to rise. Values of 3.8 mmol/L (< 70 mg/dl) are referred to as a hypoglycaemic attack (low blood sugar).

The main lipid found in the blood, bile, and brain tissues is cholesterol and it is one of the most important steroids which is a precursor of many steroid hormones of the body (Siedel et al., 1981). Esterified cholesterol is present in two thirds of the blood. The metabolized cholesterol in the liver is transported by lipoproteins in the blood stream (Flegg et al., 1973). The reported abnormal lipid disorders in diabetics are hyperlipidemia, atherosclerosis, etc. (Friedewald et al., 1972; Nelson et al., 2008). The important diagnostic of serum lipids include very low density lipoprotein, triacylglycerol, high density lipoprotein, total cholesterol etc.; thus, one of the reasons for premature atherosclerosis in persons with diabetes mellitus is an abnormal lipid metabolism (Khanna et al., 1996).

The medical community is still facing the challenge of managing diabetes without any side effect. Presently there are several drugs available to treat diabetes mellitus which include thiazolidinediones, biguanides and sulphonylurea (De-Fronzo et al. 1997). These drugs usage are restricted by their secondary failure rates, pharmacokinetic properties and accompanying side effects (Donath et al., 2006). Hence, there is an essential need for the search for a new class of compounds to overcome diabetes problems; ultimately it leads to a continuous search for an alternative medicine (Hansotia et al. 2005). *N. latifolia* for example may provide the useful dietary adjunct to existing therapies or as source for the development of pharmaceutical entities (Pepato et al., 2005). This research will reveal the efficacy of the fractions of *N. latifolia* on the lipid profile and blood sugar of diabetic rats and also establish the fraction with the highest hypoglycaemic and hypolipidemic property.

MATERIALS AND METHODS

Plant material's collection and identification

The fresh leaves of *N. latifolia* were collected at the Pharmacy farm, University of Uyo, Akwa Ibom state, Nigeria, in February, 2016. The identification and authentication of the plant were made by Dr. (Mrs.) Eshiet a Botanist in the Faculty of Pharmacy, University of Uyo, Nigeria. The voucher number deposited at the herbarium was 679. Dust particles and debris were removed from the leaves by rinsing them severally with clean tap water and then allowed to drain completely. Then, the leaves were cut, chopped into pieces, air-dried and extracted.

Plant extract's preparation

The preparation of the ethanol extract was done using the wet method of extraction: A kilogramme of the fresh leaves was cut and chopped into pieces on a chopping board using a knife and an electric blender was used to blend it in 1.5 L of 96% ethanol. It was transferred into an amber colored bottle and kept cool at 4°C in a dark compartment for 72 h. Thereafter, it was filtered with a cheese material and then Whatman No 1 filter paper was used to obtain a homogenous filtrate. A rotary evaporator was used at 37- 40°C to concentrate *in vacuo* this filtrate to about one tenth the original volume. The concentrates were dried in a water bath at 40°C, while in an open container to yield 78.95 g of brown oily substances of *N. latifolia*. A dessicator filled with self-indicating silica gel was used to completely dry it and then refrigerated at 2-8°C until needed. The residue was spread out on a clean white cardboard paper and allowed to air-dry until ethanol was completely removed.

Preparation of the fractions

Fractionation was done using the gradient method. Air-dried crude extract residue (500 g) of the *N. latifolia* were macerated using 2.5 L of N-hexane in a 5 L capacity glass ware. It was made air-tight, intermittently shaken while standing for 72 h. Whatman No.1 filter paper was used to filter the mixture and concentrated *in vacuo* using a rotary evaporator at 37-40°C to give one tenth the original volume. The water bath at 40°C was used to dry the concentrate which was left open to yield 8.3 g of an oily brown N-hexane leaf fraction of *N. latifolia*.

The residue was air-dried to completely remove n-hexane and then macerated in 2.5L of ethyl acetate for 72 h with occasional agitation. The filtrate of the filtered marc was concentrated *in vacuo* with a rotary evaporator at 37-40°C also to one tenth its original volume. The water bath at 40°C was used to dry the concentrates while open; yielding 7.95g ethylacetate leaf fraction of *N. latifolia*. The residue obtained was

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left to air dry, after which it was macerated in 2.5 L methanol in a glass ware capacity of 5 L, made air-tight, intermittently shaken and left standing for 72 h. The rotary evaporator at 37-40°C was used to concentrate *in vacuo* the filtered mixture to about one tenth its original volume. Finally, the water bath at 40°C was used to dry the concentrates while allowing open yielding 44.35 g methanol leaf fraction of *N. latifolia*.

The desicator filled with a self-indicating silica gel was used to completely dry the different concentrated fractions of the plant. They were transferred into a beaker, corked using aluminum foil and then kept at 2-8°C in a refrigerator until they are required for usage.

Experimental animals

Sixty six Albino Wistar rats; two-three weeks of age, weighing (140-150g) were purchased from the Department of Biochemistry, University of Calabar, Nigeria. The animals were kept inside a stainless steel wire cages, in well-ventilated Faculty of Pharmacy animal house, University of Uyo, Nigeria strictly under a standard environmental and adequate nutritional conditions (12 h light : 12 h dark cycle; 25 to 30°C; 35-60% humidity) throughout the period of experiment. Feeding with commercial feed and drinking water of the animals were done *ad libitum*. The animals were left to acclimatize for fourteen days before commencement of the study. The ethical guide for care and use of laboratory animals of the University of Uyo, Nigeria were strictly adhered to throughout the experiments.

Diabetes induction

Sixty overnight fasted rats were injected intraperitoneally with 150 mg /kg alloxan (Sigma, St. Louis, Mo, USA) for diabetes induction. Hypoglycemia was prevented in the induced animals by placing them on a 10% glucose solution in the next 24 h of induction (Jarald et al., 2008). Hyperglycemia were recorded for 48 rats with fasting blood glucose (FBG) > 200 mg/dl) on the fourth day of diabetes induction and thus inculcated into the diabetic group of the study.

Experimental design

Eight groups of diabetic animals and a group of normal non-diabetic animals were used as shown in Table 1. The fractions were orally administered twice daily for 14 days, while glibenclamide (a reference drug) was administered once daily, and also orally. Blood glucose of individual rat was monitored at every 24 h interval. After the end of 14-days treatment, the rats were fasted overnight and anaesthetized in the morning with chloroform. Blood was collected through cardiac puncture and kept for 2 hours to clot. The whole blood was centrifuged at 6000 rpm for 20 min to separate out the serum. Lipid profile assay was done using the sera.

Biochemical analysis

The level of blood glucose (BGL) was obtained using Accucheck ActiveTM on strips of glucose in Accu-check ActiveTM test meter with the blood obtained via the tail vein of fasted rats. Enzyme method of Asanga et al. (2013) was used to assay the level of total cholesterol (TC), Heber et al. (2013) enzymatic colorimetric method was used to determine the triglyceride (TG), while the phosphotungstate method of Tripathi et al. (2012) was applied in the assay of high density lipoprotein (HDL-C); all kits were of Agappe laboratory. Very low density lipoprotein concentration (VLDL-C) and low density lipoprotein concentration (LDL-C) were extrapolated using the values obtained for TC, TG and HDL; thus, VLDL-C = TG/5, while LDL-C = TC - (HDL-C + VLDL-C) (Friedewald et al., 1972).

Analysis of data

Data are reported as mean ± standard error of mean (S.E.M). One-way analysis of variance (ANOVA) and the Duncan's post hoc test were used in analyzing the results. Statistically, significant was considered at P = 0.05.

RESULTS AND DISCUSSION

Treatment effect on concentrations of blood glucose

Significant differences (P=0.05) were observed in the concentrations of blood glucose in the negative control (394.66±84.47mg/dl) in comparison with that of the positive control (49.33±16.2 mg/dl) and normal control (84.00±3.98 mg/dl). All the plant fractions indicated a significant difference (P=0.05) in levels of blood glucose (150 mg/kg N-hexane =150.50±31.44 mg/dl, 300 mg/kg N-hexane =134.25±28.62 mg/dl, 150 mg/kg ethyl acetate =163.75±93.65 mg/dl, 300 mg/kg ethyl acetate = 368.67±79.82 mg/dl, 150 mg/kg of methanol =116.75 ± 4.92 mg/dl, 300 mg/kg of methanol 138.00 ± 14.01 mg/dl) when compared with all the controls as shown in Table 2.

Treatment effects on lipid profile

All the lipid profiles had a significant differences (P=0.05) in the negative control (TG=122.79±21.65 mg/dl, TC=383.76±79.68 mg/dl, HDL-C=41.72±0.61 mg/dl, VLDL-C=42.58±10.35 mg/dl, LDL-C=299.46±79.23 mg/dl) when compared with the positive (TG=89.24±14.30 mg/dl, TC=116.36±14.69 mg/dl, HDL-C=66.45±0.21 mg/dl, VLDL-C=17.85±2.86 mg/dl, LDL-C=32.06±13.23 mg/dl) and normal controls (TG=181.43±7.17 mg/dl, TC=97.97±12.78 mg/dl, HDL-C=35.09±5.93 mg/dl, VLDL-C=36.51±1.49 mg/dl, LDL-C=24.64±12.70 mg/dl), respectively. Also, all the fractions: 150 mg/kg-ethyl acetate (TC=69.88±8.52 mg/dl, LDL-C=8.23±7.76 mg/dl), 300 mg/kg ethyl acetate (TC=51.08±9.93 mg/dl, LDL-C=6.44±7.66 mg/dl), 150 mg/kg N-hexane (TC=69.88±8.52 mg/dl, LDL-C=8.23±7.76 mg/dl), 300 mg/kgN-hexane (TC=51.08±9.93mg/dl, LDL-C=6.44±7.66mg/dl) 150 mg/kg-methanol (TC=116.86±13.34 mg/dl, LDL-C=50.68±14.13 mg/dl), and 300 mg/kg-Methanol (TC=108.66±12.77 mg/dl, 42.09±79.93 mg/dl) had a significant differences (P=0.05) on LDL-C and TC in comparison with the controls. except the 150 mg/dl methanol TC which was similar to the positive control. For VLDL-C, the fractions: 150 mg/kg-ethyl acetate=34.94±1.84 mg/dl), 300 mg/kg ethyl acetate (=22.24±2.77 mg/dl), 150 mg/kg-methanol =35.13±2.78 mg/dl), and 300 mg/kg-methanol=33.76±3.17 mg/dl) indicated a significant differences (P=0.05) when compared with the positive and negative controls, while

Table 1. The designed experimental study.

Group	Quantity of rats	Pre-treatment	Treatment
1 = Normal control	6	Non-alloxan treated rats (normal rats)	Distilled water
2 = Negative control	6	Alloxan treated	Distilled water
3 = Positive control	6	Alloxan treated	5 mg/kg glibenclamide
4=N-hexane fraction	6	Alloxan treated	150 mg/kg N-hexane fraction
5=N-hexane fraction	6	Alloxan treated	300 mg/kg N-hexane fraction
6=Ethylacetate fraction	6	Alloxan treated	150 mg/kg Ethyl acetate fraction
7=Ethylacetate fraction	6	Alloxan treated	300 mg/kg ethyl acetate fraction
8=Methanol fraction	6	Alloxan treated	150 mg/kg methanol fraction
9=Methanol fraction	6	Alloxan treated	300 mg/kg methanol fraction

Table 2. Treatment effect of blood glucose concentration.

S/N	Group	Treatment	Blood glucose concentrations		
			Starting value (mg/dl)	Ending value (mg/dl)	Percentage change (%)
1	Normal control	Distilled water	132.50±7.59	84.00±3.98*[b]	36.60*,[b]
2	Negative control	Distilled water	296.00±16.62	394.66±84.47[a,b]	33.33,[a,b]
3	Positive control	5 mg/kg Gliberclamide	589.00±10.02	49.33±16.2*,[a]	91.62*,[a]
4	N-hexane fraction	150 mg/kg N-hexane fraction	288.50±18.37	150.50±31.44*,[a,b]	47.83*,[a,b]
5	N-hexane fraction	300 mg/kg N-hexane fraction	374.75±14.27	138.00±14.01*,[a,b]	64.17*,[a,b]
6	Ethylacetate fraction	150 mg/kg ethyl acetate fraction	310.75±20.45	163.75±93.65*,[a,b]	44.5*,[a,b]
7	Ethylacetate fraction	300 mg/kg ethyl acetate fraction	365.66±20.35	134.25±28.62*,[a,b]	0.82.*,[a,b]
8	Methanol fraction	150 mg/kg of methanol fraction	281.00 ±16.09	116.75 ± 4.92*,[a,b]	58.45*,[a,b]
9	Methanol fraction	300 mg/kg of methanol fraction	385.25 ±19.72	368.67±79.82*,[a,b]	64.18*,[a,b]

Data were expressed as mean ± SEM, n = 6. *P = 0.05: compared with Negative Control; [a]P = 0.05: compared with normal control; [b]P = 0.05: compared with positive control, that is, Glibenclamide.

they were similar to that of the normal control. There was a significant difference (P=0.05) in the positive control in the levels of HDL in comparison with the normal and negative controls (Table 3).

DISCUSSION

Induction of diabetes with alloxan presented significantly raised blood glucose by 3-5 times its normal values in non-diabetic rats. Research carried out by Kahn et al. (2005) and Henry et al. (2005) on the fractions of *Ocimum sanctum* and *N. latifolia,* respectively in diabetic and non-diabetic rats produced similar result as seen in this work. Contrary, the 14-day treatment with 150 mg/kg N-hexane, 300 mg/kg N-hexane, 150 mg/kg ethyl acetate, 300 mg/kg ethyl acetate, 150 mg/kg methanol fractions and glibenclamide presented significant decrease (P=0.05) in blood glucose of diabetic animals by 58.45%, 64.18%, 47.83%, 64.17%, 44.50% and 91.62% of their initial values, respectively as shown on Table 2. Some researchers (Asanga et al., 2013; Effiong and Essien, 2014) also gave similar report on the ability of fractions of *N. latifolia* in reducing blood glucose concentrations. Research carried out by Kahn et al. (2005) on the fractions of *O. sanctum* on normal and diabetic rats also produced similar result as in this research.

The hypoglycemic effects of ethyl acetate and N-hexane fractions decreases with dose increase as their 300 mg/kg dose gave a greater decrease as compared to their 150 mg/kg dose. On the other hand, the blood glucose concentration was significantly increased (P=0.05) when treated with 300 mg/kg-methanol fraction as it gave a 0.82% decrease from the initial value in comparison with the negative control group. Animals of the negative control group were still hyperglycemic (368±79.82 mg/dl). They may have developed diabetic ketoacidosis, a diabetic emergency, which is usually characterized by extreme hyperglycemia. This occurs due to the absence of insulin, possibly caused by severe pancreatic beta cell damage by the alloxan.

The significant hypoglycemic activity of N-hexane, ethyl acetate and methanol (150 mg/kg) fractions could be an

Table 3. Treatment effect of lipid profile.

S/N	Group	Treatment	Lipid profile (mg/dl)				
			TG	TC	HDL-C	VLDL-C	LDL-C
1	Normal control	Distilled water	181.43±7.17*,b	97.97±12.78*	35.09±5.93	36.51±1.49b	24.64±12.70*
2	Negative Control	Distilled water	122.79±21.65a	383.76±79.68a,b	17.87±3.35a,b	42.58±10.35a,b	299.46±79.23a,b
3	Positive Control	5mg/kg Gliberclamide	89.24±14.30a	116.36±14.69*	66.45±0.21±,a	17.85±2.85*,a	32.06±13.23*
4	N-hexane fraction	150mg/kg N-hexane fraction	174.73±9.19*,b	69.88±8.52*	26.70±1.17*,b	34.94±1.84b	8.23±7.76*,a,b
5	N-hexane fraction	300mg/kg N-hexane fraction	111.17±13.87a	51.08±9.93*	22.41±3.75*,a,b	22.24±2.77*,a	6.44±7.66*,a,b
6	Ethylacetate fraction	150mg/kg Ethyl acetate fraction	175.62±13.91*,b	116.86±13.34*	31.06±2.67b	35.13±2.78b	50.68±14.13*,a
7	Ethylacetate fraction	300mg/kg Ethyl acetate fraction	166.91±15.44*,b	108.66±12.77*	32.78±5.24b	33.76±3.17*,b	42.09±9.93*
8	Methanol fraction	150mg/kg of Methanol fraction	110.36±3.47	73.16±18.62*,b	41.72±0.61*a,b	22.04±0.6*	33.24±16.19*
9	Methanol fraction	300mg/kg of Methanol fraction	148.59±6.53*	69.78±8.41*,b	33.08±6.80b	29.21±1.00*	9.29±5.62*,b

Data were expressed as mean ± SEM, n = 6. *P = 0.05: compared with negative control; aP = 0.05 : compared with normal control; bP = 0.05: compared with positive control, that is, Glibenclamide. TC = total cholesterol, TG = Triacylglyceride, HDL-C = high density lipoprotein-cholesterol, VLDL-C = very low density lipoprotein cholesterol, LDL-C = low density lipoprotein cholesterol.

effect of flavonoids, triterpenes and saponins present in *N. latifolia* leaf (Gidado et al., 2008; Asanga et al., 2013). The plant extracts may also contain some biomolecules that could sensitize the insulin receptor to insulin or stimulate the islets of Langerhans beta cells to release insulin; thereby, enhancing the carbohydrate metabolizing enzymes to re-establish an improved blood glucose level (Umar et al., 2010; Asanga et al., 2013).

Increased levels of VLDL, LDL-C, TG and TC are seen in the negative control rats accompanied by insulin resistance, heart disease, diabetes mellitus because the peripheral fat deposits mobilized more fatty acids from it (Bopanna et al., 1997; Nelson et al. 2008); these were decreased after treatment with *N. latifolia* fractions which could be their ability to arrest some diabetes mellitus symptoms related to lipid and its suggestion for the disease management. However, there were significant reductions (P = 0.05) of LDL-C concentrations in most fractions and glibenclamide in comparison with the negative control, and which is similar to an earlier report by Nikkila (1984) and Asanga et al. (2013). LDL-C concentration in the blood has positive correlation with incidence of cardiovascular diseases. Also, there were significant reduction (P = 0.05) in the concentrations of VLDL-C in the glibenclamide treated group, 300 mg/kg of ethyl acetate fraction compared with the normal and negative controls. Similar result were earlier reported by Dhandapani et al. (2002) and Akah et al. (2009).

Flavonoids, phenols, saponins and sterols which are present in *N. latifolia* have been reported by Katsumata et al. (1999) to be accompanied with hypocholesterolemia and hypolipidemia. This suggest the reason for the obtained result in N-hexane and ethylacetate particularly, the 300 mg/kg ethyl acetate fraction which had the greatest effect in TG, TC, VLDL-C and LDL-C concentrations in comparison with the negative control as seen in Table 3. Furthermore, an improved result was noted in all the fractions as compared to the glibenclamide treated group which is a reference drug for diabetes in lowering total cholesterol and LDL-C, as N-hexane showed a significant difference, while the difference in ethylacetate was not significant (P ≠ 0.05).

Conclusion

Decreased total cholesterol, low density lipoprotein-cholesterol and fasting blood glucose concentrations with improved level of high density lipoprotein by the fractions indicates that, the fractions have hypoglycaemic and hypolipidemic activity. These effects observed in the fractions were dose dependent as the higher doses produced a more significant effect as seen in Tables 2 and 3. From the study, ethyl acetate fraction may be the best agent in arresting hyperglycemia and hyperlipidemia arising from diabetes mellitus followed by N-hexane and then

methanol. And thus, affirms the rationale for their usage to treat diabetic.

CONFLICTS OF INTERESTS

The authors have declared no conflict of interests.

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Antiproliferative and pro-apoptotic effects of Andean berry juice (*Vaccinium meridionale* Swartz) on human colon adenocarcinoma SW480 cells

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***Vaccium meridionale* Swartz, commonly Andean Berry, has a high content of several phytochemicals, such as anthocyanins, phenolic acids, and other flavonoids. However, in spite of its antioxidant capacity, there is little information about its anticarcinogenic properties. This study evaluated the antiproliferative and pro-apoptotic activity of Andean Berry Juice (ABJ) on human colon adenocarcinoma SW480 cells. The antiproliferative activity of ABJ was evaluated on SW480 cells using the Sulphorodamine B assay.The effect on cell viability, cytotoxicity and activation of caspase-3 was analyzed using The ApoTox-Glo™ Triplex Assay. Specific apoptotic biomarkers cleaved PARP, total Bcl-2-associated death promote (BAD), phosphorylated BAD, total p53, and phosphorylated p53 were also analyzed. To determine the intracellular redox-state, the Glutathione Assay Kit and 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) were used, respectively. The antiproliferative assay showed a IC50 value of 8% v/v ABJ, the caspase 3 activity was increased in time-dependent manner in SW480 treated cells, the proapoptotic proteins (cleaved caspase 3, cleaved PARP, P53 and total BAD) were increased by 1.6 to 2.0 fold. In addition, the ABJ-treated SW480 cells increased significantly the production of intracellular reactive oxygen species (ROS), parallel with reduction in the intracellular content of glutathione (GSH) and consequently a decrease of GSH/ oxidized glutathione (GSSG) ratio. In conclusion, the ABJ was able to inhibit SW480 cells proliferation involving apoptotic mechanisms through the perturbation of intracellular oxidative state.**

Key words: Vaccinum, colon cancer, anthocyanins, apoptosis, oxidative stress.

INTRODUCTION

According to the International Agency for Research on Cancer (IARC), approximately 663.904 new cases of Colorrectal Cancer (CRC) were diagnosed and 320.397 people died worldwide during 2008 (Boyle and Levin, 2008). The countries with the highest incidence rates include Australia, New Zealand, Canada, USA and some

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parts of Europe (Forman et al., 2014). According to epidemiological findings of Doll and Peto (1981), 35% of all deaths from CRC are associated with nutritional factors, either by the presence of dietary carcinogens, or by the absence in the diet of foods with preventive properties. The World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) concluded that, the dietary factors associated with the increase of risk for CRC are: (i) the consumption of red and processed meat, (2) low intake of foods containing fiber, milk and calcium, and (3) high intake of alcohol supported with convincing and probable evidence (Boyle and Levin, 2008).

At the same time, the IARC reported that eating foods containing fiber such as fruits, whole grain, cereals, legumes and vegetables in three servings/days, reduced the risk of CRC by 21.5% (Forman et al., 2014). In addition, it has promoted the consumption of certain types of fruits and vegetables whose antioxidant, antigenotoxic, anti-inflammatory and antiproliferative properties are able to prevent initiation of colorectal carcinogenesis, or retard this process towards to promotion stages.

There is strong evidence that the antioxidants present in fruits and vegetables protect lipids, proteins and nucleic acids against the oxidative damage initiated by free radicals (Ferguson et al., 2004). Fruit extracts of *Vaccinium* species including slowbush blueberry, bilberry, cranberry, and lingonberry inhibit multiple stages of carcinogenesis and stimulate the apoptosis of cancer cells (Kraft et al., 2005; Ferguson et al., 2016; Safrin et al., 2016). These effects may be partly attributable to polyphenolic compounds such as flavonols, anthocyanins, and proanthocyanidins contained in this genus.

Vaccinium meridionale Swartz (Andean Berry) is a Colombian native plant that belongs to the Ericacea family. This fruit has been considered as a potential functional food because of its high content of phenolic compounds and anthocyanins that attribute an antioxidant capacity similar to or higher than the values reported for other species of *Vaccinium* (Gaviria et al., 2009; Gaviria et al., 2012). The total antioxidant capacity and phenolic composition of Andean Berry was investigated by (Gaviria et al., 2009). They reported that, the content of anthocyanins and total phenols with values of 201 ± 10 mg eq/100 g of fruit and 609 ± 39 mg eq/100 g of fruit, respectively. The antioxidant activity was studied by the methodologies 1,1-diphenyl-2-picryl-hydrazyl (DPPH: 2404 ± 120 values of mM de trolox®/100 g of fruit), ABTS (8694 ± 435 values of mg de ac. Asc/100 g of fruit) and FRAP (581 ± 29 values of mg de ac. Asc/100 g of fruit), all these results are comparable or superior to other Vaccinium species published in other researches (Capocasa et al., 2008; Çelik et al., 2008).

Recently, our group reported that (Maldonado et al., 2014) the aqueous extracts of Andean Berry contain total anthocyanins: 150.7 mg of cyanidin-3-glucoside equivalents/100 g of lyophilized; total phenols: 2546 mg of gallic acid equivalents/100 g of lyophilized. Phenolic acids such as chlorogenic: 126 mg/100 g of lyophilized; ferulic: 108 mg/100 g of lyophilized, coumaric: 63/100 g of lyophilized. In addition, this extract presented a high trapping capacity of reactive oxygen species (ROS), reactive nitrogen species (RNS) and hydroxyl radical scavenging capacity: 36147.5 ± 6274.7 (µmol DMSO/100 g lyophilized), total scavenger capacity to ROS and RNS: 29255.9 ± 6531.27 µmol Trolox/100 g lyophilized), 41775.2 ± 6168.2 µmol Trolox/100 g lyophilized), respectively, and ORAC value: 41775.2 ± 6168.2 µmol Trolox/100 g lyophilized). These properties could be partially explained by the presence of the high content of anthocyanins and phenolic acids (Maldonado et al., 2014).

In this study, it was reported for the first time the proapoptotic effects of an aqueous extract (juice) of Andean Berry on colon adenocarcinoma SW480 cells. Evidence was presented that a juice of Andean Berry was able to inhibit the growth of SW480 cells by triggering apoptosis and involving the oxidative stress which favours death of SW480 cancer cells.

MATERIALS AND METHODS

Plant

Fresh ripe berries of *V. meridionale* (Andean Berry) were harvested from the Municipality of Retiro (Antioquia, Colombia), at 2175 m altitude and 16°C in May 2015. Berries were washed, selected, disinfected (sodium hypochlorite 100 ppm), dried and processed for 2 min at 2500 rpm and freeze-dried in a vacuum chamber under pressure 0.427 + 0.5 mm Hg, at a temperature of -50°C, after lyophilization the powder was stored at room temperatura (RT) and protected from light in PET packaging aluminium, to be used as an ingredient in the subsequent preparation of the juice.

Preparation of Andean berry juice

Andean Berry Juice (ABJ) was prepared as described previously by Franco-Tobón et al. (2016). In brief, freeze-dried powder of Andean Berry was dissolved in sterile water and sucrose to obtain a juice of 11.1° Brix, acidity 4.33 mg citric acid/ml, pH 3.06. Firstly, the juice was prepared. Further, the homogenized samples (juice) were kept in a 2.0 ml micro centrifuge tubes and sonicated at different time intervals of 15, 30, 45 and 60 min at room temperature (25 ± 1°C). The ultrasonic treatment was performed using an ultrasonic cleaner (42 kHz, 135 W; Branson ultrasonic corporation, USA). The sonicated product was aliquoted and stored at -70°C, protected from light until it was used for cell treatments.

Cell culture

SW480 cells were cultured as described by Maldonado et al. (2014). In Brief, a 15 cm^2 Falcon flask with Dulbecco's modified eagle's medium was supplemented with 25 mM glucose, 2 mM L-glutamine, 10% heat (56°C)-inactivated horse serum, 100 U/ml

penicillin, 100 µg/ml streptomycin, and 1% no-essential amino acids. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. For all experiments the ITS medium (10 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium) was used and the horse serum was reduced to 3%.

Antiproliferative sulforhodamine B (SRB) assay

This assay is based on staining of total cellular protein from cells with SRB dye and performed as described Gossé et al. (2005). Briefly, SW480 cells were seeded in 96-well culture plates at a concentration of 2×10^3 cells per well and cultured at 37°C in 5% CO_2. After 24 h seeding, the cells were exposed to different concentrations of the ABJ (3, 6, 8, and 10% v/v per well) and incubated for different times (24, 48, and 72 h). Cell layers were fixed to the well bottoms by adding 50 µl of 10% trichloroacetic acid (TCA) in each well, and the plates were incubated at RT for 1 h. The wells were then drained, rinsed twice with distilled water, and air dried. SRB (0.4% w/v in 1% glacial acetic acid) was then added (100 µl/well), and the plates were incubated for 30 min. Unbound dye was drained and removed by washing 3 times with 1% glacial acetic acid. After air-drying the plate overnight, the dye was solubilized by adding 100 µl/well of 10 mM Tris base and stirling for 10 min. Absorbance at 520 nm was measured in a GloMax®-Multi+ Microplate Multimode Reade (Molecular Devices; Sunnyvale, CA, USA). All experiments were performed in triplicate. The concentration able to kill 50% of cells (IC50) was calculated using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The absorbance of control group (non-treated cells) was considered as 100% viability. The percent inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - (ODt / ODc)] \times 100$$

ODt is the optical density (OD) of treated cells and ODc for control (non-treated cells).

Cell viability, cytotoxicity and caspase 3 activity

The SW480 cells were plated in 96-well micro plates (2×10^3 cells/well). After 24 h, the cells were incubated during 48 h, at 37°C, 5% CO_2 at different concentrations of ABJ (3, 6, 8 and 10% v/v). The effect of juice on SW480 cell viability, cytotoxicity and activation of caspase-3 was analyzed using The ApoTox-Glo™ Triplex Assay kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Briefly, 10 µl of viability/cytotoxicity reagent containing both glycylphenylalanyl-aminofluorocoumarin (GF-AFC) substrate and bis-alanylalanyl-phenylalanyl-rhodamine 110 (bis-AAF-R110) substrates were added to all wells. After incubation for 1 h at 37°C, the Relative Fluorescence Units (RFU) was determined at 505 nm excitation/400 nm emission for cell viability and at 520 nm excitation/485 nm emission for cytotoxicity. To determine the effect on caspase-3 activity of SW480 cells treated or not with the Andean berry juice, 10 µl of caspase- substrate DEVD-sequence complexed with luciferase, which will be hydrolyzed by caspase-3 activated to generate a luminic signal produced by luciferase, thus the luminance is proportional to the amount of Andean Berry juice-activated caspase 3 in SW480 cells, the Relative Luminiscence Units (RLU) from the caspase-3 activated was measured after 30 min of incubation. The fluorescence and luminiscence was detected using a GloMax®-Multi+ Microplate Multimode Reader (Molecular Devices; Sunnyvale, CA, USA).

Apoptosis analysis

To determine the apoptotic effect of ABJ on SW480 cells, the PathScan® Apoptosis Multi-Target Sandwich ELISA Kit (Cell Signaling Technology, Masachusset, USA) was used. Briefly, antibodies for detect cleaved caspase 3, cleaved PARP, total BAD, phosphorylated BAD, total p53 and phosphorylated p53, had been coated onto microwells by the manufacturer. After incubation with lysates from treated SW480 cells during 48 h, with 8% v/v of Andean berry juice, the target protein was captured by the coated antibodies. Following extensive washing, a detection antibody was added to detect the captured target protein. An Horseradish Peroxidase (HRP)-linked secondary antibody was then used to recognize the bound detection antibody, after TMB HRP-substrate was finally added for blue color development which was proportional to the quantity of bound target protein, color reaction was stopped by an acidic stop solution and optical density was analyzed at 450 nm in GloMax®-Multi+ Microplate Multimode Reader (Molecular Devices; Sunnyvale, CA, USA).

Glutathione assay

The SW480 cells were cultured as describe earlier and treated with the ABJ at 3, 6, 8 and 10% v/v final concentrations per well for 48 h, 37°C and 5% CO_2. To determine the antioxidant cell status after treatment, the GSH Glo® Glutathione Assay Kit (Promega, WI, USA) was used following the manufacturer's instructions. Birefly, 50 µl of GSH-Glo® Reagent were incubated for 30 min in 24-well opaque microplates containing the treated and non-treated cells, before adding 100 µl of Luciferin Detection agent. Following 15 min incubation, Glutathione Reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of NADPH. Subsequently, the chromogen reacts with the thiol group of GSH to produce a colored compound that absorbs at 405 nm. The plates were read in a GloMax®-Multi+ Microplate Multimode Reade (Molecular Devices; Sunnyvale, CA, USA). The GSH content was calculated from a GSH standard curve. The luminiscence data were reported as RLU. GSH and GSSG in SW480 cells after treatment were then calculated and the ratio of GSH/GSSG was calculated by the following formula:

$$\text{Ratio GSH/GSSG treated} = [\mu M\ GSH – (\mu M\ GSSG \times 2)]/\mu M\ GSSG.$$

Statistical analysis was performed according to student's t-test by one way analysis of variance. Significant difference was taken as $p<0.05$. Each reported value was the mean ± SD from 3 independent experiments.

Determination of intracellular ROS

The 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) is one of the most widely used to measured directly the redox-state of a cell. The DCFH-DA is a cell permeable, nonfluorescent precursor of DCF that can be used as an intracellular probe for oxidative stress. The intracellular esterases cleave DCFH-DA at the two ester bonds, producing a relatively polar and cell membrane-impermeable product, the H2DCF. This nonfluorescent molecule accumulates intracellularly and subsequent oxidation yields the highly fluorescent product DCF. Cells were seeded in culture dishes (2×10^4 cells per 2.5 cm internal diameter) and treated with ABJ at 3, 6, 8 and 10% v/v final concentrations per well for 48 h, 37°C and 5% CO_2, cells were resuspended in pre-warmed PBS containing 8 µM DCFH-DA and incubated for 30 min at RT in darkness. For positive control, H_2O_2 (1.05%, v/v) was added to cell culture 15 min before read. Data were presented as Relative Fluorescence Units (RFU) signal, measured at 520 nm excitation/485 nm in a G GloMax®-Multi+ Microplate Multimode Reade (Molecular Devices; Sunnyvale, CA, USA).

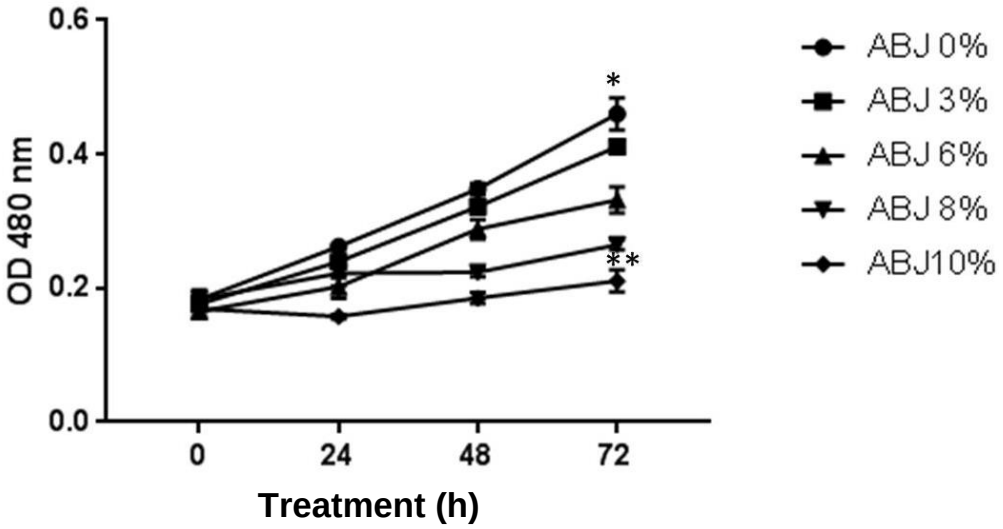

Figure 1. Antiproliferative effects of ABJ at different concentrations on the growht of SW480 cells after 24, 48 and 72 h of treatment. Data correspond to the means ± SD of three independent experiments. ANOVA was performed for comparing between groups, Dunnett's multiple comparisons as post-test. The asterisk indicates a significant difference between non-treated cells and treated ($p<0.05$).

Statistical analysis

The data were presented as mean ± standard error (SE) from three independent experiments. Comparisons between groups were done by one- and two-way ANOVA. Comparison between treated and not treated with Andean berry juice was done by two-tailed paired t-test. Results were considered significant when $p<0.05$. These analyses were done with the GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego California, USA).

RESULTS

Effect of ABJ on SW480 cell growth

The growth inhibitory effects of ABJ on SW80 cells were tested using the colorimetric method of sulforhodamine B (SRB) as described by Gossé et al. (2005). Figure 1 shows that cells treated at 3, 6, 8 and 10% ABJ for 24, 48 and 72 h, the SW480 cell proliferation was inhibited in a concentration-dependent manner. After 48 h, a significant inhibition of SW480 cell growth ($p <0.05$) at 6, 8 and 10% ABJ compared to non-treated cells was observed. This effect was observed until 72 h of cells exposed at 8% ($p<0.004$) and 10% ($p<0.0034$) of ABJ compared to non-treated cells under the same conditions. The IC50 at 24, 48 and 72 h was 19, 8 and 3% of ABJ, respectively.

Effect of ABJ on SW480 cell viability, cytotoxic and activation of caspase 3

Figure 2 shows the effect ABJ on SW480 cell viability, cytotoxic effect and caspase 3 activation after treatment at 8% ABJ (IC_{50} for 48h). The SW480 cell viability was reduced in a time-dependent manner whereas the cytotoxic effect evidenced by the increase on the RFU values corresponding to dead or dying cells were increased in time (Figure 2). Under the same conditions, the activation of caspase-3 was paralled to cytotoxic effect until 48 h. After 72 h of 8% ABJ treatment, the caspase-3 activity fell-down which suggests that most cells are dead by apoptosis and only a small number of these are dying by the apoptosis triggered by ABJ during all the treatment.

Effect of ABJ on apoptotic biomarkers in SW480 cells

Figure 3 shows that ABJ'IC_{50} induces phosphorylation of p53 at Ser15 with significant statistical differences ($p= 0.04$) compared with untreated cells, as well as ABJ´IC_{50} induces cleavage of PARP and caspase-3. In the other hand, it has already been established that phosphorylation of Bad at Ser 112 inactivates its proapoptotic function. Our findings suggest that ABJ´IC_{50} does not induce Bad phosphorylation; this may lead the increase of apoptotic-promoting activity of Bad and thereby contribute to the death of this human colon cancer cell line.

Intracellular ROS level and GSH depletion assay

Ratio of reduced glutathione (GSH) and oxidized

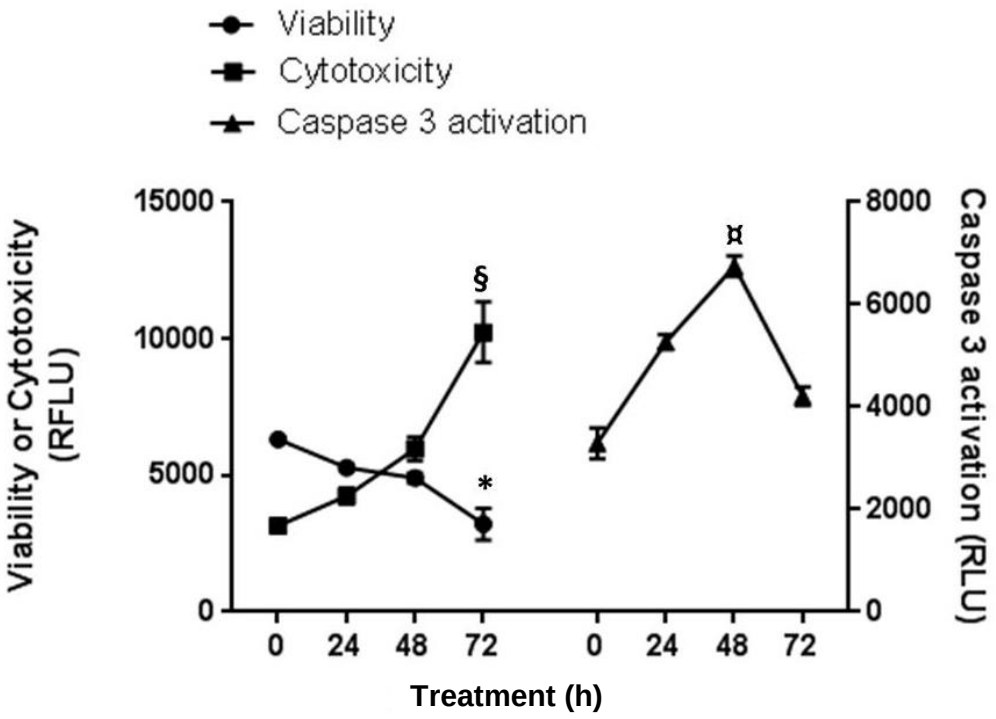

Figure 2. Effect of ABJ on SW480 cell viability, cytotoxicity and caspase 3 activation. SW40 cells were exposed for 24, 48 and 72 h with 8% ABJ. Data are the mean ± SD of three separate experiments. Symbols *, § and ¤ denote statistical significance in comparison with untreated cell controls using a one-way ANOVA (P<0.05) by Tukey's mean comparison.

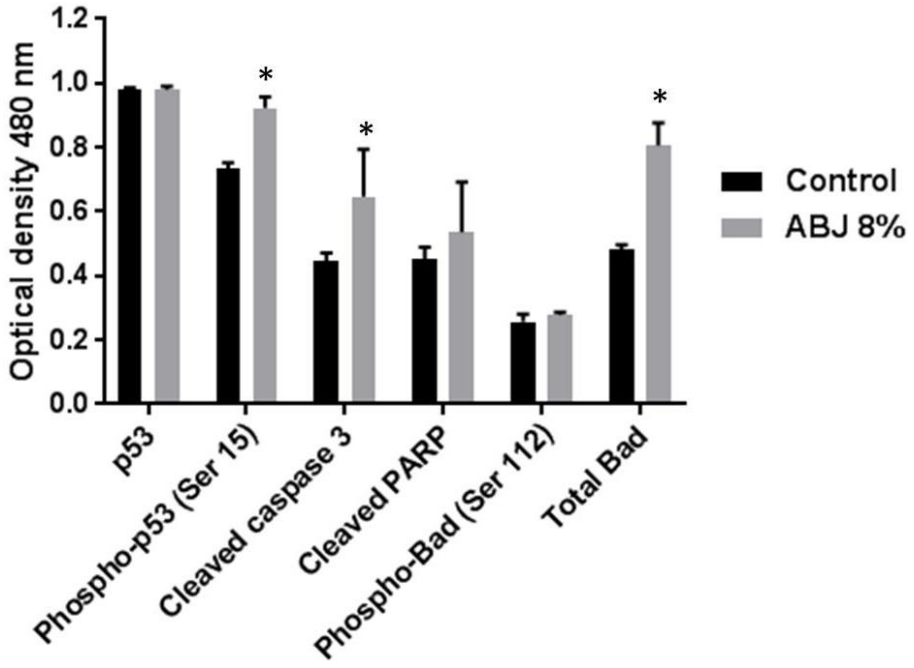

Figure 3. Effect of ABJ on apoptotic biomarkers in SW480 cells. The SW480 cells were treated at 8% ABJ for 48 h as described in materials and methods section. Data correspond to the means ± SD of three independent experiments. ANOVA was followed by Dunnett's multiple comparisons test. The asterisk indicates significant (p<0.05) differences between controls (black bar, untreated cells) vs. treated cells (gray bars).

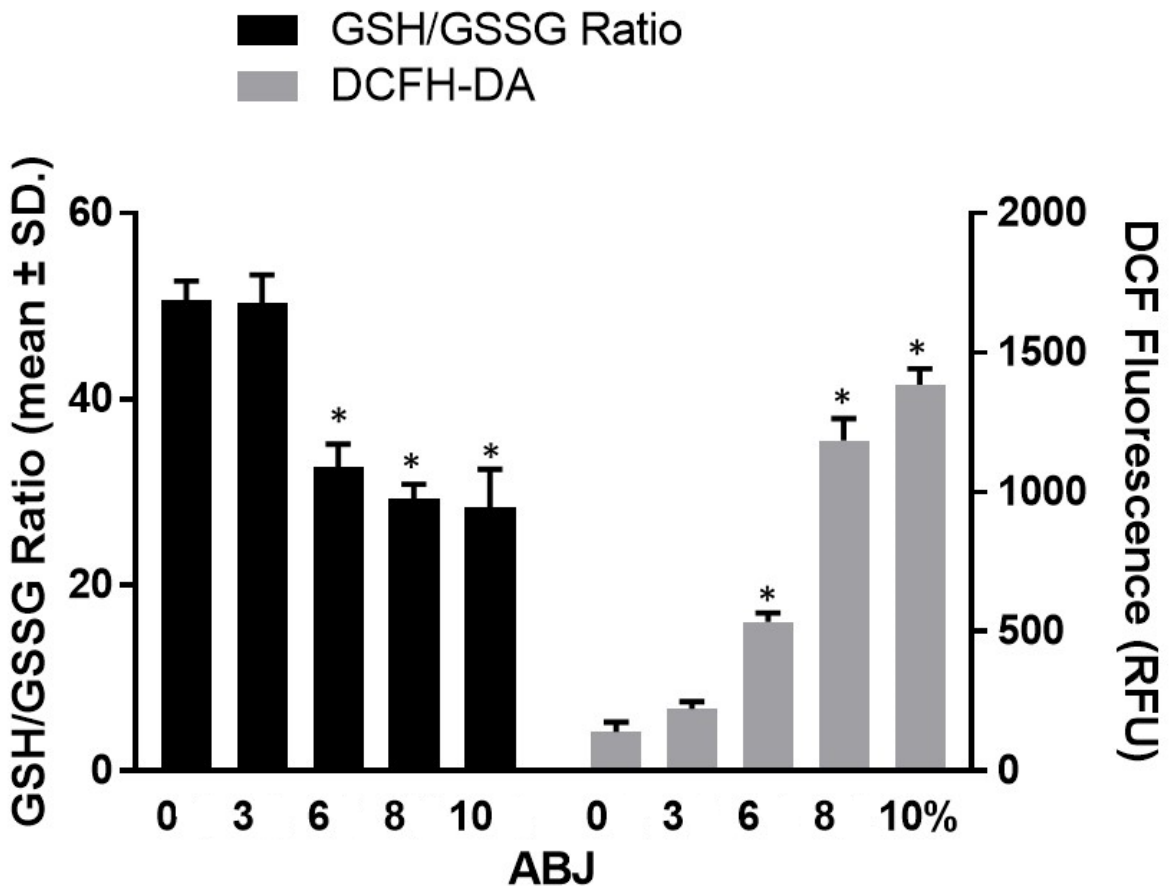

Figure 4. The plot shows the ratio of GSH (reduced form)/GSSG (oxidized form) and the ROS DCF level (mean ± SD, n = 3) upon treatment with ABJ at 3, 6, 8 and 10% v/v, against SW480 cells for 483dr h. Significant difference (∗) was taken as $p<0.05$ in comparison with untreated control cells.

glutathione (GSSG) was measured in SW480 cells after 48 h of incubation after treatment with ABJ. Increased levels of oxidized form of glutathione (formed as results of ROS scavenging) are indicative of oxidative stress. As can be seen from Figure 4, ABJ produced significant glutathione depletion as evident by the increased formation of GSSG particularly at the concentration of 8% v/v compared to vehicle control.

Figure 4 shows the effect of ABJ on modulation of the intracellular oxidative state of SW480 cells after 48 h of incubation, the intracellular ROS production was also measured by DCF method. The ABJ (from 3 to 10% v/v) induced a significant dependent-increase of intracellular ROS at 6% v/v in SW480 cells. Maximum increase ROS level was detected at concentration of >8% v/v of ABJ.

DISCUSSION

In this study, the antiproliferative and proapoptotic effects of an Andean berry juice on colon adenocarcinoma cell line SW480 was evaluated. These juices contain most variety of the berry phytochemicals which allows to do an evaluation of their anticarcinogenic capacities (Franco et al., 2016). Although we have previously reported the free radical scavenging capacity of *Vaccinium meridionale* Sw freeze-dried aqueous extract by chemical methods and the antiproliferative effects against colon cancer cell lines (Maldonado et al., 2014). In our study, by the first time, we show that this juice is a potential anticancer agent able to eliminate a colon cancer cell (SW480) through apoptosis involving the stress oxidative as mechanisms involved in these events.

It was found that ABJ had a IC_{50} value of 8% of fresh juice v/v, an effect also observed in similar studies evaluating the anticancer effects of 13 edible berries on 5 cancer cell lines (AGS stomach adenocarcinoma, MFC-7 and MDA-MB-231 mammary gland, PC-3 prostatic adenocarcinoma and Caco2 colorectal adenocarcinoma cell line) using juices made with a domestic extractor (Boivin et al., 2007). In that study, the authors found that most berries juices reduced the proliferation of cancer cells lines, but the extent of inhibition was different between the various berries juices, for example intestinal

cancer cell line Caco-2 was inhibited by Cranberry, Raspberry and Blackcurren, but was much less susceptible by gooseberry and sea buckthorn than other cell lines (Boivin et al., 2007). They found a IC_{50} value of 25% v/v of Cranberry juice for colon cancer Caco-2 cells, higher than the IC_{50} of ABJ on SW480 cells. This discrepancy could be explained by the fact that *Vaccinium meridionale* Swartz contain high levels of antioxidants, approximately 1-fold, that other species of berries as Cranberrie (Mosquera et al., 2015).

In the other hand, Bermúdez Soto et al. (2007) reported that incubation of Caco 2 cells with a pre-digested chokeberry juice at a nontoxic dose (final pH 7.5 and osmolarity 325 miliosmoles L-1 in the culture medium) increased the cytotoxicity (20%) and reduced cell proliferation (30 to 40%). They also found with the use of microarrays and RT-PCR. An increase in the expression of tumor suppressor genes such as CEACAM1 and BMP2 (2.6 and 2.4 fold, respectively). In turn, they reported downregulation of genes related to tumor invasion and metastasis FGFR2 and S100A4 (-4.6 and -2.5 fold, respectively) in response to juice treatment (Bermúdez et al., 2007).

All these data support our results of the antiproliferative and proapoptotic effects observed here with an Andean berry juice on colon adenocarcinoma cells.

Apoptosis is a complex process that proceeds through at least two major pathways (intrinsic and extrinsic) which are regulated at multiple levels. The uncontrolled cell division and suppressed apoptosis are main characteristic features of cancer cells (Kang et al., 2006; Vidya et al., 2010).

Caspases are a group of proteins belonging to the group of cysteine proteases. Caspases are essential mediators of the apoptosis. One of them is the caspase 3 that hydrolyzes DEVDG (Asp-Glu-Val-Asp-Gly) peptide and can activate caspase 7 favouring a DNA damage that would inevitably lead to cell suicide. Caspase 3 is an apoptosis-executing protein and can be activated by either two major pathways (Lemaire et al., 1998).

One of the possible mechanisms that could explain the death of carcinogenic cells in the presence of ABJ could be the apoptotic processes, so we decided to determine if the SW480 cells exposed to the ABJ treatment induce the activation of caspase 3. It was found out that a time-dependent increase manner in caspase 3 activity in response to the IC_{50} ABJ treatment which indicates that carcinogenic cell death is the result of an apoptotic process, because Caspase 3 is an executioner caspase and an important apoptotic biomarker. This proapoptocic effect on colon adenocarcinoma cells like Caco-2, SW480 and HT-29 with increase in caspase 3 activity has been reported for other berries juices and extracts such as Cranberry, blueberry, raspberry and acai berry (Safrin et al., 2016; Tamara et al., 2014). In our study, the ABJ juice does not only induced an increase in caspase 3 activity of SW480 cells but in the activation of others proapoptotic biomarkers such as the PARP cleavage fragment. The primary function of PARP-1 is to detect and repair DNA damage, however, cells with profound DNA damage amplify PARP-1 activity, in such a way that ATP deposits are depleted, resulting in a passive necrotic cell death, Caspases can block this process, thanks to the cleavage of PARP-1 which is considered as a distinctive sign of apoptosis (Eguchi et al., 1997).

On the contrary, Boivin et al. (2007) found that the death of colon adenocarcinoma cells Caco-2 exposed to different berries juices had no correlation with the increase in caspase 3 activity. These discrepant results can be explained by some differences present in both studies such as: (I) the use of different juices berries; Boivin et al. (2007) used a juice from Cranberry, Raspberry and Blackcurren made with a domestic extractor, while in this study, we used a juice from Freeze-dried powder of Andean berry, (II) the used of different adenocarcinoma cells lines ($CaCo_2$ and SW480 cells, respectively), and III) different periods of incubation; they incubate for a period of 24 h and was done in a period of 48 h.

In order to determine the possible apoptotic mechanisms involved in the apoptotic effects observed in SW480 cells treated with 8% ABJ, the endogenous levels of p53 protein, phospho-p53 (Ser15), total Bad, phospho-Bad (Ser112), and cleaved poly (ADP-ribose) polymerase-1 (PARP) were measured. These molecules represent key signaling proteins in pathways controlling survival and apoptosis.

Severe oxidative stress induces apoptosis followed by DNA damage, p53 is phosphorylated and translocated to the nucleus where it triggers multiple mechanisms including modulation of the Bcl-2 family proteins, amplification of death signals and activation of Caspases (Thompson et al., 2004). In our study, it was observed that ABJ enhanced the activation of tumor suppressor protein p53 in SW480 cells; it was also found that ABJ upregulated the expression of tumor Bcl2 family proapoptotic protein Bad. This protein is regulated through its phosphorylation. Dephosphorylated Bad promotes apoptosis by binding to Bcl-2 family, while phosphorylation of Bad at Ser75 in a MAPK-dependent manner facilitating its inactivation. Promoting cells survival, in our study, it was found that the phosphorylated form of Bad was inhibited in the cells exposed to ABJ compared to control cells as well as the cell treated with ABJ, induced the proteolytic cleavage of caspase 3. Activation and execution of the apoptotic pathway in SW80 cells was also perceptible by increased level of cleaved PARP (Figure 5). However, we cannot determine which one of the two major apoptosis pathways is involved, since we did not make caspase 8 (apoptosis extrinsic pathway) or caspase 9 (apoptosis intrinsic pathway) determinations.

In a recent study, Carole Minker et al. (2015) identified which proapoptotic pathway is induced in human colon

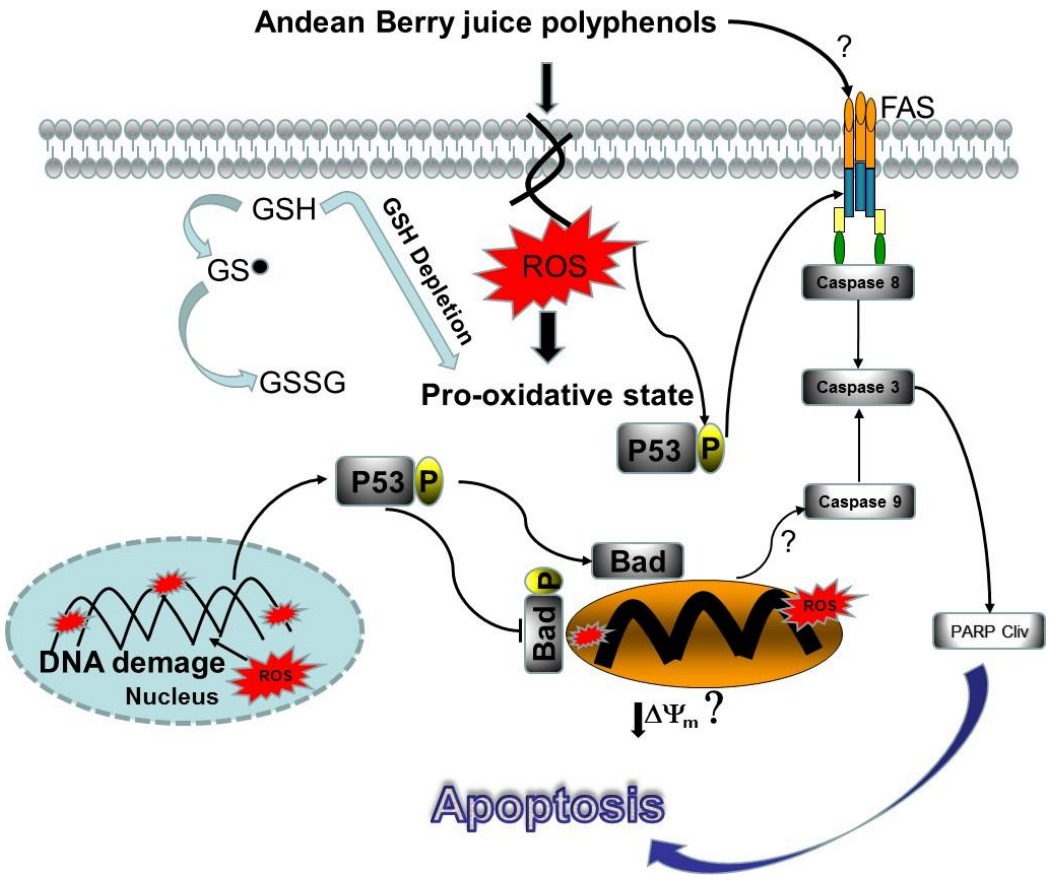

Figure 5. Scheme of a proposed apoptosis-induced signaling pathway triggered by ABJ in SW480 cells. ABJ induces intracellular ROS overproduction in a time depend manner, with increase of GSSG. The accumulation of intracellular ROS may lead to a DNA damage-mediated p53 activation and co-regulate the expression of Bad, a pro-apoptotic member of Bcl-s family proteins which form a heterodimer with anti-apoptotic proteins and favors the apoptosis through the mitochondria disruption that consequently may activate a caspase cascade and PARP cleavage.

cancer cell lines (SW480 and SW620), exposed to proanthocyanidins (Pcys) extracted from 11 berries. They found that Lowbush blueberry extract triggers the strongest activity of all berries tested by the opposite blueberry. Pcys are less effective for DNA fragmentation. Finally, they concluded that Lowbush blueberry Pcy-induced apoptosis is mediated by TNF-R1, DR3, or DR6, these death receptors inducing caspases 8 and 9 activation (Minker et al., 2015). Further studies are in course in our group to gain more insight into the apoptotic mechanisms triggered by ABJ on colon cancer cell lines and in a preclinical model of colon carcinogenesis.

The ROS production was measured in the SW480 cells exposed to ABJ using DCHFDA, as shown in Figure 4, the ABJ induce ROS production in a concentration dependent manner, even ten-fold higher compared with untreated cells. This increase in ROS levels occurred when a decrease in GSH/GSSG ratio was also observed in ABJ-treated SW480 cells, indicating that SW480 cells are under a condition of oxidative stress during the apoptotic process, in spite of the antioxidant capacity previously reported and measured using free-cell living methods such as ORAC (Maldonado et al., 2014).

The concentration of ABJ used here on SW480 cells induced oxidative stress which may be involved in the apoptotic process because an increase of intracellular ROS may lead to a DNA damage able to activate the p53, in addition, the increase of ROS production may also activates the release of cytochrome c from mitochondria and favours the activation of caspse 9 previous to activation of caspase 3 (Pelicano et al., 2004; Lamy et al., 2008) demonstrated that apoptosis of SW480 cells treated with a flavonoid identified as lupulone at 40 μg/ml for 48 h was associated to the increase of ROS intracellular concentration involving release of cytochrome c, caspase 9 and caspase 3 activation. These effects were avoided when SW480 cells were exposing to vitamin C in the presence of lupulone (Lamy et al., 2008). Thus, these observations suggest that ABJ might activate in SW480 cells the intrinsic

apoptotic pathway; however, this hypothesis must be confirmed in further studies.

Recently, Khan et al. (2014) proposed that the fact that carcinogenic cells have higher levels of copper compared with non-malignant cells and may be more susceptible to the transfer of electrons with antioxidants to generate ROS, so damage to DNA by copper may be an important route by which carcinogenic cells can die in the presence of polyphenols, while normal cells can survive (Azmi et al., 2006).

In conclusion, the present study shows a novel insight into the mechanism of action of ABJ induced apoptosis on human colon adenocarcinoma cells. A link was reported between apoptosis cell death and the increase of intracellular ROS, our date supported the potential of ABJ as a chemotherapeutic agent, an anti-proliferative activity for human colon adenocarcinoma.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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