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Research Article

In vitro anti-inflammatory and anticoagulant activities of alkaloïds extracted from nopals of inermis Algerian *Opuntia ficus indica (L)*.

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Abstract

The dearth of information surrounding the utilization of *Opuntia* cladode alkaloids underscores a critical gap in understanding their pharmacological properties and therapeutic potential, emphasizing the need for further comprehensive investigations. The present study aims to investigate the in vitro anti-inflammatory, anticoagulant, and antimicrobial activities of alkaloïds belonging to young cladodes (nopals) of inermis Algerian *Opuntia ficus indica*. The assessed alkaloïds showed a moderate anti-inflammatory effect regarding the BSA protein protection with a maximum of 51.04±1.84 % compared to 84.22±2.38 % for Diclofenac sodium as a positive standard. However, their stabilization of red blood cells membrane against induced hemolysis was greater than Diclofenac (52.38±2.01 % vs 48.97±2.73 %). The two assays had a significant correlation (< 0.05) of 0,968. Nopal alkaloïds extended the coagulation time (1.24 fold) by significantly affecting the exogenous pathway PT only, whilst they were ineffective against the endogenous pathway APTT. Conversely, Algerian nopal alkaloïds had neither bacteriostatic nor bactericide influence at 2 mg ml^{-1} on Candida albicans yeast or the six tested pathogenic bacterial strains. In perspective, the purification of these *Opuntia* nopal alkaloïds and understanding their accurate mechanism of action are clearly the strategic steps to illustrate their overall curative potential.

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1. Introduction

The *Opuntia ficus-indica* (L.), or prickly pear cactus, is a subtropical or tropical plant belonging to the Cactaceae family. This species is commonly dispersed in arid and semi-arid climates of the Mediterranean and Central America, to which it presents a high morpho-physiological adaptation capacity to their extreme environmental conditions (Stintzing & Carle, 2005). The thick and succulent paddle-like parts of this cactus are named cladodes. They are spiny or spineless, oblong with variable widths, and possess a waxy, water-proof epidermis with a photosynthesis capacity and asexual reproduction (Heuzé & Tran, 2017). They have recently received a growing attention among researchers and economics worldwide for their multivalent nutritional and pharmaceutical potentials (Belhadj Slimen et al., 2016). The young edible cladodes called "nopals" are viewed as functional foods or medicinal plants owing to their large amount of active ingredients, including polyphenols, flavonoids, carotenoids, steroids, and alkaloïds (Stintzing & Carle, 2005; Suryawanshi & Vidyasagar, 2016). These natural compounds offer a valuable means to enhance food quality and prolong its shelf-life without the need for unwanted chemical preservatives while also find applications in cosmetics and pharmaceuticals (Ramli et al., 2020). Alkaloïds are bio-cyclonitrogenic molecules, attributed most often to the same family of plants as a consequence of similar biosynthetic pathways (Yubin et al., 2014). Despite the high toxicity of many of these compounds, their significant pharmacological activity justifies their wide use in plant medicine or as pharmaceuticals (Aruwa et al., 2018). Inflammation and coagulation are two interplayed vital host defense mechanisms that work together to combat invading pathogens, minimize tissue damage, and restore homeostasis within the body. Inflammation triggers coagulation activation, and coagulation in turn influences inflammatory processes. Pro-inflammatory cytokines and other mediators can activate the coagulation system while down-regulating key physiological anticoagulant pathways. Conversely, activated coagulation proteases can interact with specific receptors on inflammatory and endothelial cells, thereby modulating the inflammatory response (Lazzaroni et al., 2021). As the information available on the biological activity of Algerian inermis nopal alkaloïds is quite maigre, the fixed objective of this study is to explore their in vitro anti-inflammatory, anticoagulant, and antimicrobial aspects in order to get a broader comprehension into their pharmacological properties against pathologic inflammatory conditions, clotting disorders, and infectious diseases.

2. Materials and methods

2.1 Chemicals and reagents

Alkaloïds extract

The alkaloïds' dry powder of spinless Tissemsilt-Algeria nopals was granted from the Laboratory of Beneficial Microorganisms, Functional Food and Health (LMBAFS), Faculty of Natural Sciences and Life, University of Abdelhamid Ibn Badis, Mostaganem, Algeria. This dry extract, giving a yield of 0.59 %, contains seventeen identified alkaloïd as reported by our previous work (Moussaoui et al., 2022).

Reagents

The analytical grade: hydrochloric acid (37 %), sodium chloride (NaCl), calcium chloride ($CaCl_2$), Dimethyl sulfoxide (DMSO), and sodium citrate ($Na_3C_6H_5O_7$), in addition to Brain Heart Infusion Broth (BHIB), Sabouraud medium and Mueller Hinton Agar were provided from Sigma Aldrich (St. Louis, MO, USA). The biochemical reagent grade: Diclofenac sodium, PT reagent, APTT

reagent, and lyophilized Bovine Serum Albumin (BSA) (≥ 98.0 %) were purchased from Merck Millipore (Darmstadt, Germany).

Microbial strains

The yeast strain Candida albicans ATCC (10231), along with bacterial pathogenic strains Klebsiella pneumoniae (ATCC 13883), Staphylococcus aureus (ATCC 33862), Proteus mirabilis (ATCC 35659), Pseudomonas aeruginosa (ATCC 27853) and Bacillus cereus (ATCC 10876), as well as the commensal strain Escherichia coli (ATCC 25922) belong all to the American Type Culture Collection ATCC.

2.2 Anti-inflammatory activity

Evaluation of anti-arthritic effect

This quality was measured using the "inhibition of protein denaturation" method. Three aliquots (0.45 ml) of an aqueous Bovine Serum Albumin solution (BSA) (5 % w/v) divided on tubes were added with 0.05ml of alkaloïds extract (15.75-250 µg ml^{-1}), distilled water, and Diclofenac sodium (15.75-250 µg ml^{-1}) to give the test solution, the control test solution and the standard solution respectively. The product control solution is a mixture of 0.45 ml of distilled water added to 0.05 ml of test solution. The above solutions were adjusted by HCl (1 N) solution to pH = 6.3. The tubes were incubated at 37 °C / 20 min then kept at 57 °C for 3 min. After cooling, 2.5 ml of phosphate buffer solution PBS (pH = 6.3) was added to each tube. The absorbance was monitored spectrophotometrically at 416 nm, and the inhibition of protein denaturation was calculated by the formula (1):

$$\% \text{ Inhibition} = 100 - \left[\frac{Abs\ Test\ solution - Abs\ Product\ solution}{Abs\ Test\ control} \right] . 100 \qquad (1)$$

The negative control showed 100 % of BSA denaturation, while Diclofenac sodium served as a positive control.

Inhibition of hemolytic effect on red blood cells (HRBC Method)

Blood samples (approximately 6 mL) were collected from healthy volunteers without steroidal treatment during the last two weeks. After centrifugation at 3000 g / 5 min, the cells pellet was subsequently recovered and washed with an equivalent volume of normal saline (pH 7.4), then centrifuged again. The cleaning step was repeated several times until obtaining a clear supernatant. The human red blood cells were prepared with normal saline to get a suspension of 10 % (v/v) and used immediately. The effect of alkaloïds on the stabilization of erythrocyte membrane was carried out by mixing 1 mL of the alkaloïds' extract at a range (15.75-250 µg mL^{-1}) to 1 mL of PBS (0.15 M, pH 7.4) and 2 mL of the hyposaline solution (0.25 % NaCl). The obtained 4.5 mL mixture was completed with 0.5 mL of 10 % erythrocyte suspension. The isotonic saline replaced the extract in the blood control, while it substituted the erythrocyte suspension in the drug control. The mixture tubes were heated at 56 °C /30 min, cooled for 20 min under running water and centrifuged at 3000 rpm/5 min. The absorbance of the supernatants was read at 560 nm against a buffered NaCl solution as a blank. The positive standard was the Diclofenac sodium. The stabilization of the membrane was calculated by the formula (2) of Oyedapo and Famurewa (1995):

$$\% \text{ Membrane stability} = 100 - \left[\frac{Abs\ of\ test\ drug - Abs\ of\ drug\ control}{Abs\ of\ blood\ control}\right] \quad (2)$$

The control gave 100 % erythrocyte membrane lysis (0 % stability).

2.3 Anticoagulant activity

These assays were carried out by the method of Athukorala et al. (2007):

Plasma preparation

The blood samples drawn by venipuncture from healthy donors without issues of thrombosis or bleeding were added to sodium citrate (3.8 %) in a ratio of (9:1). Normal pooled plasma was made by centrifugation for 20 min at 2400 x g and then stored at (-60 °C) until use.

Activated partial thromboplastin time assay (APTT)

The mixture made by 90 µl of prepared plasma and 10 µl of alkaloïds solutions in different concentrations (31.25-250 µg mL^{-1}) was warmed at 37 °C for 1 min, and then added by 100 µl of APTT reagent. After incubation at 37°C for 5 min, the coagulation was triggered by adding 100 µl of $CaCl_2$ (0.025 M) and APTT was recorded. The substitution of samples by 0.9 % NaCl and heparin gave the negative and positive controls, respectively.

Prothrombin time (PT) assay

The mixture of 90 µl of prepared plasma and 10 µl of alkaloïd solutions at a range of (31.25-250 µg mL^{-1}) was incubated at 37 °C/10 min. The clotting time was recorded after adding 200 µl of reagent PT pre-incubated for 10 min at 37 °C. The NaCl solution (0.9 %) and heparin replaced the alkaloïds in the negative and positive controls.

2.4 Antimicrobial Activity

The sensitivity of six pathogenic bacterial strains and one yeast strain towards nopal alkaloïds was evaluated. According to the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeasts testing, bacterial strains were revitalized prior to utilization at 37 °C for 18 hours in BHIB medium. The yeast was reactivated in Sabouraud medium for 48 hours at 25 °C. Inoculums were adjusted to 10^8 CFU mL^{-1} for bacteria and 10^6 CFU mL^{-1} for yeast, corresponding to an optical density (600 nm) ranging between 0.08-0.1, and 0.12-0.15, respectively (Balouiri et al., 2016).

Agar well diffusion method

The Mueller Hinton plate surface was inoculated overly by spreading 100µL of the previous fresh bacterial or yeast suspensions. After 15 minutes, holes of 6 mm diameter punched aseptically in the seeded agar were filled with 50 µl of 2 mg mL^{-1} nopal alkaloïds extract dissolved in Dimethyl sulfoxide (DMSO). The plates underwent pre-incubation at 4 °C for 2 hours, allowing a radial diffusion of the extract, followed by incubation for 48 hours at 25 °C for yeast and 24 hours at 37 °C for bacteria. Antimicrobial activity was inferred by measuring the diameters of inhibition zones surrounding the wells (Balouiri et al., 2016; Ramli et al., 2020).

Determination of minimum inhibitory concentration (MIC)

The MIC was deduced using the microdilution method in a liquid medium as cited by Sánchez et al. (2014). Wells of 96-well microplates containing 100 µL of each microbial suspension (10^6 CFU mL^{-1}, final concentration) were supplemented with 100 µL of nopal extracts (2 mg mL^{-1}, maximum concentration) serially diluted geometrically at a ratio of 2. Wells with only culture medium or with bacterial suspension without nopal extract were considered as controls. Plates were incubated in the same previous conditions for yeast and bacteria. MIC was the lowest concentration of extract that inhibited the visible growth of bacteria (Balouiri et al., 2016).

Determination of minimum bactericidal and fungicidal concentrations (MBC and MFC)

MBC and MFC were established by seeding an aliquot of 25 µL from wells having a concentration equal to or greater than the MIC on appropriate agar plates. They were distinguished as the lowest concentrations of alkaloïds at which no visible microbial growth was detected on the plates (Balouiri et al., 2016).

2.5 Statistical analysis

The results of triplicate measurements were presented as means ± S.D. The student's t-test and the Analysis of variance ANOVA was performed using IBM SPSS statistics (version 26), and the p-value was considered as significant when (< 0.05). Correlation analysis using Pearson's coefficients was conducted between the independent assays and the alkaloïds concentrations on one hand and between the independent assays on another hand.

3. Results and discussion

3.1 Anti-inflammatory effect

Serum bovine albumin denaturation

Protein denaturing is a common process in inflammation and arthritis. By involving the loss of structure and configuration of proteins, denaturation causes the loss of their functionality and leads to auto-antigen formation in vivo (Alam et al., 2015). The prevention of this phenomenon presents an interesting property that is much sought after in anti-inflammatory drugs. According to the results presented in (Figure. 1A), the inhibition of BSA denaturation by the alkaloïds increased with concentration to reach a maximum of 51.04±1.84 % at 62.50 µg mL^{-1}, after which the inhibition decreased proportionally to the alkaloïds concentration. The anti-inflammatory drug (sodium Diclofenac) exhibited a higher but non-significant positive effect ($p > 0.05$) in a dose-dependent manner, noted 84.22±2.38 % at the highest dose of 250 µg mL^{-1}. Vinchurkar et al. (2014) linked the anti-denaturation effect of proteins to the presence of alkaloïds. Similarly to our results, other scientific studies showed the highest BSA protection at small concentrations and lower protein preservation activity with extract than standard drug (Tatti et al., 2012). Since inflammation and oxidation are interrelated, the neutralization of free radicals by alkaloïds can attenuate the inflammation response. Consequently, the pro-inflammatory effect of alkaloïds at high concentrations can be explained by the intensification of their NO_2, $COCH_3$, and COOH groups in the reaction media, capable according to Al-Sehemi and Irfan (2017) of attracting an electron instead of losing it in a pro-oxidizing act.

Membrane stabilization

To evaluate the anti-inflammatory effect of nopal alkaloïds, the membrane stabilization of human erythrocytes test was performed. The use of erythrocyte membrane as a model is argued by their structural resemblance to the membrane of lysosomes involved in the inflammatory reaction (Alamgeer et al., 2015). The protective effect of alkaloïds of Opuntia nopals and positive standard (Diclofenac) against induced hemolysis is shown in (Figure 1B). The alkaloïds were able to inhibit the hemolysis in a non-dose-dependent manner with 52.38±2.01 % as a maximum value and 27.21±1.45 % as a minimum value, noted at 62.5 µg mL^{-1} and 130 µg mL^{-1}, respectively. Thereby, these alkaloïds capable of protecting the erythrocyte membranes can stabilize the lysosomes, and inhibit the release of their constituents to limit the inflammatory response. This mechanism is the acting mean of certain non-steroidal anti-inflammatory agents and herbal preparations (Drăgan et al., 2016). When measured against alkaloïds, Diclofenac showed a weaker anti-hemolytic activity ($p > 0.05$) in a dose-dependent mode, giving an interval between 18.36±1.26 % and 48.97±2.73 %; the most effective concentration was 250 µg mL^{-1}. The concentration-dependent activity of Diclofenac was confirmed by Ghumre et al. (2017). It can be proposed that nopal alkaloïds acted by modifying the superficial charge to prevent interaction with aggregating agents, as they contributed to the regulation of water exchange by protecting the membrane proteins forming ion channels (Oyedapo et al., 2010). In addition, they scavenge free radicals responsible for membrane lipid peroxidation (Jayakumar et al., 2016). According to the previous observation made by Moussaoui et al. (2022) about the pro-oxidant effect of Algerian nopal alkaloïds at high concentrations, a comparable rate of activity was observed in the inhibition of BSA denaturation and hemolysis, with an increase of positive effect to a maximum peak at 62.5 µg mL^{-1} followed by a remarkable regression. It can be confirmed that the pro-oxidant effect proposed at high doses had a direct pro-inflammatory action.

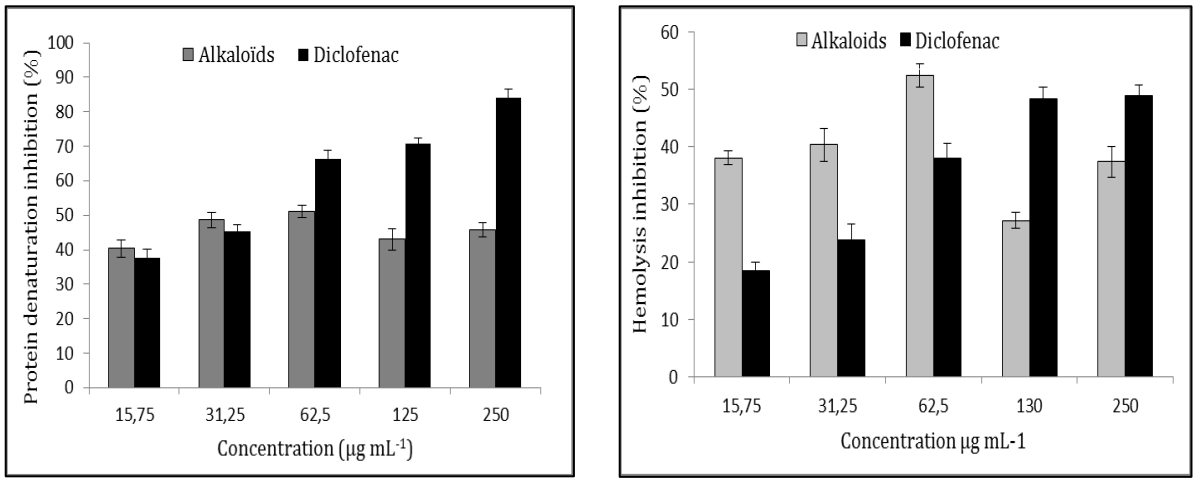

Figure 1. Anti-inflammatory effect of alkaloïds and Diclofenac sodium against Bovin serum albumin denaturation (A) and Human erythrocytes hemolysis (B)

3.2 Anticoagulant activity

The effect of nopal alkaloïds on the exogenous and endogenous coagulation pathways, using PT and APTT in vitro assays, is mentioned in (Figures 2A and Figure 2B). The APTT test indicates the effect of alkaloïds on factors of the intrinsic pathway, while PT assesses the extrinsic coagulation pathway (Khouya et al., 2015). The samples and heparin prolonged significantly ($p < 0.05$) the control PT (12.13±1.45 s), with an obvious and significant ($p < 0.05$) superiority for heparin, which lengthened the coagulation by 11.42 times to give 138.64±8.27 s, compared to 1.24 times

for the alkaloïds with PT equal to (15.11±1.94 s), at the highest concentration (250 µg mL^{-1}). However, the alkaloïd samples didn't show any prolongation of the APTT time and even reduced it to (25.86±8.79 s) as a minimum below the control time (30.1±3.88 s), suggesting a pro-coagulant effect by stimulation of the endogenous factors. The heparin kept the same positive rate ($p < 0.05$) by extending the APTT control time to (627.98±13.21 s) with a doubling effect of 20.86 times at 250 µg mL^{-1}. It can be suggested that nopal alkaloïds were ineffective towards the intrinsic clotting pathway but blocked the exogenous one, while heparin affected both with a preferential action to the endogenous and/or common pathway. The presence of sugar residues and sulfates in molecular structure was indicated to be responsible for anticoagulant activity (Chaouch et al., 2018). The lack of these moieties in the identified alkaloïds, except for Glucobrassicin, which contained one sulfate group, was hypothesized as an explanation for their weak anticoagulant effect.

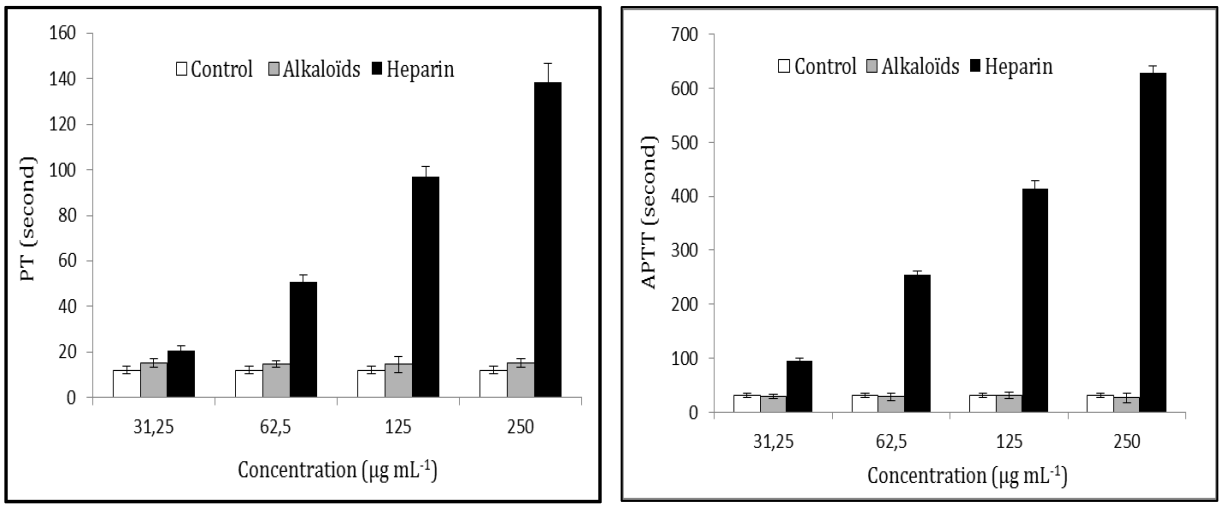

Figure 2. Anticoagulant activity of alkaloïds and heparin on Prothrombin time (PT) (A) and Activated partial thromboplastin time (APTT) (B)

3.3 Antimicrobial Activity

The agar well diffusion method performed in this work is extensively used to assess the antimicrobial ability of vegetal extracts, where they diffuse in the agar medium and deter the development of the strain tested. Similarly, dilution methods are still the most fitting techniques for determining quantitatively the lowest concentration of the examined antimicrobial agents able to inhibit the visible growth of the microorganism tested, named MIC (µg mL^{-1} or µg L^{-1}) (Balouiri et al., 2016). Likewise, the most common valuation of bactericidal or fungicidal activity remains the determination of the minimum lethal concentration (MLC), usually known as minimum fungicidal or bactericidal concentrations (MFC and MBC). Their values are deduced by sub-culturing an aliquot from wells characterized by a negative microbial growth during the assessment of MIC to calculate the number of living cells (CFU mL^{-1}) after being incubated for 24 h. They refer to the lowest antifungal/antimicrobial agent concentration required to kill 99.9 % of the final inoculum (Balouiri et al., 2016). Algerian nopal alkaloïds were ineffective at the 2 mg mL^{-1} tested concentration against all strains, as no inhibition zones were obtained (Table 1). This inefficiency witnessed the limitation of nopal alkaloids as anti-bacterials. The low concentration used could likely be the cause of the absence of antimicrobial effect. Aruwa et al. (2019) found that the concentrations required to exert antimicrobial activity are higher for phyto-molecules than those derived from bacteria and fungi. According to Aruwa et al. (2019), MICs for different solvent nopal extracts ranged between 25 mg mL^{-1} as a minimal threshold and 300 mg mL^{-1} for all bacteria tested,

including the same strains of this work, for instance, Bacillus cereus (ATCC 10876), Staphylococcus aureus (ATCC 29213), Pseudomonas aeruginosa (ATCC 27853), Klebsiella pneumonia (ATCC 700603), Escherichia coli (ATCC 25922). This extract's lack of antibacterial activity should be verified by using much higher concentrations. Nonetheless, the inexistence of molecules with potent antimicrobial effect in the alkaloïds extract may be another argument justifying the absence of antimicrobial activity. Molecules like Berberine, piperine, and reserpine, demonstrating a respective inhibitory effect on streptococcal, E. coli, and K. pneumoniae proliferation in the research of Barbieri et al. (2017), were not identified in the seventeen alkaloïds identified in our previous work (Moussaoui et al., 2022). Moreover, the presence of antimicrobial active principles in mixtures can probably attenuate their activity, seeing that certain constituents may exert an antagonistic effect or even nullify the positive action of bioactive agents (Dos Santos et al., 2015). All pure alkaloïd compounds (yohimbine, vincamine, scopolamine, atropine, colchicine, allantoin, trigonelline, octopamine, synephrine, and capsaicin) assayed by Özçelik et al. (2011), have possessed sturdy antimicrobial properties against similar strains such as C. albicans ATCC (10231), P. mirabilis ATCC (7002), B. subtilis ATCC (6633), S. aureus ATCC (25923), K. pneumonia RSKK (574), P. aeruginosa ATCC (10145) and E. coli ATCC (35218). Hence, the antibacterial activity is strongly linked to the extraction solvent of antibacterial materials, the dose used likely more effective when increased, and the type of tested bacteria where Gram-negative bacteria are more resistant compared to gram-positive bacteria due to their distinct morphological structure and composition (Bhuyar et al., 2020).

Table 1. Antimicrobial activity, MIC, MBC, and MFC of nopal alkaloïds used at a maximal concentration of 2 mg mL^{-1}

Strain	Inhibition zone (mm)	MIC (mg mL^{-1})	MBC or MFC (mg mL^{-1})
	Gram +		
Bacillus cereus ATCC 10876	-	nd	Nd
Staphylococcus aureus ATCC 33862	-	nd	Nd
	Gram -		
Pseudomonas aerugenosa ATCC 27853	-	nd	Nd
Escherichia coli ATCC 25922	-	nd	Nd
Proteus mirabilis ATCC 35659	-	nd	Nd
Klebsiella pneumoniae ATCC 13883	-	nd	Nd
	Fungus		
Candida albicans ATCC 10231	-	nd	Nd

nd: non-determined

3.4 Correlation between alkaloïds, anti-inflammatory, and anticoagulant assays

The Pearson's coefficient was adopted to evaluate the correlation between the alkaloïds concentrations and the anti-inflammatory and anticoagulant activities. From Table 2, the exogenous coagulation pathway PT was the unique parameter affected positively following the increase in alkaloïds' concentration. The correlation alkaloïds-APTT was the most negative (-0.601), describing an acceleration of the endogenous clotting process (Table 2). Both protection of BSA protein and inhibition of erythrocyte membrane destruction were negatively correlated to the augmentation of alkaloïds rates, proposing a better inflammation appeasement at low doses of nopal alkaloïds. A good protection of proteins promotes higher membrane stabilization, expressed by a significant pearson's coefficient (< 0.05) up to 0,968.

Table 2. Correlation coefficients (r) between alkaloïds concentration and anti-inflammatory and anticoagulant assays, and between different assays

	Alkaloïds concentration	BSA protection	Membrane stabilization	APTT	PT
BSA protection	-0,559^{ns}	1			
Membrane stabilization	-0,383^{ns}	0,96816801*	1		
APTT	-0,601^{ns}	-0,273443518	-0,3859732	1	
PT	0,222^{ns}	0,182516627	0,10398232	-0,689772353	1

*significant at *p*-value < 0.05, ns: non-significant

4. Conclusion

The versatility of *Opuntia* nopals in industry and cosmetology has been firmly affirmed in recent decades. However, its main use remains limited to their consumption as food eaten cooked or raw, or even as by-products. This work enlightens the hidden therapeutic bioactivity of Algerian nopals by exploring the potential in vitro biological activities of their alkaloïds. The results have led to the fact that nopal alkaloïds can reduce the amplitude of the inflammatory reaction to a certain degree by preserving membrane integrity, as they can intervene by hindering the transformation of proteins into antigens. Their positive impact on the coagulation process was also present but restricted to the exogenous pathway. Inversely, these cactus metabolites were ineffective at the used dose against pathologic bacterial and yeast strains. The omnipresent implication of nopal alkaloïds in various interrelated pathological mechanisms can be enormously useful in healing diseases such as arthritis and cardiovascular or neurodegenerative dysfunctions. In perspective, supplementary experiments are needed to purify and highlight the mechanisms by which nopal alkaloïds modulate these interrelated biological processes.

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Author's declaration

The authors have no competing interests to declare. MB (Lecturer) Conceptualized idea, designed research methodology, executed the experiment, collected and analyzed data, interpreted data and wrote the manuscript. HT (PhD student) Literature review, helped design research methodology, data collection and interpretation. RA (Lecturer) Contributed to the conceptualization of the study, experimental design and data interpretation, and contributed to statistical analysis. GL (Lecturer) Statistical analysis and literature review. RB (Laboratory engineer) Performed experiment, prepared figures, literature review, and data interpretation. AH (Laboratory engineer) Performed experiment, prepared figures, literature review, and data interpretation. ZK (Zemour Kamel) Plant identification, literature review, language proofreading. RA (Professor) Conceived idea, designed research methodology, edited and gave the final approval of the manuscript. All authors read and approved the final version of the manuscript.

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