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Chemical composition, *in vitro* anti-inflammatory and antioxidant properties of chloroform and ethyl acetate extracts from *Pardoglossum Cheirifolium* (L.) Barbier & Mathez.

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Abstract

This study aimed to investigate the chemical composition (by GC-MS& HPLC), *in vitro* anti-inflammatory (protein denaturation method) andantioxidant (DPPH, *β*-carotene) activities of both chloroform (PCC) and ethylacetate (PCEA) extracts obtained from the aerial parts of the Algerian *Pardoglossum cheirifolium* (L.) Barbier & Mathez. The subjection of PCC to GC-MS analysis resulted into the identification of seventeen metabolites of which we cite2-ethylheptacosane (15.02%), pentacosane (12.12%) and tetracosane (10.59%) as the major components. The examination of PCEA byHPLC analysis leaded to the identification and quantification of seventeen phenolic compounds including nine flavonoidsand eight phenolic acids with epicatechin(106.5625 mg/g) and 2-hydroxycinnamic acid (46.687 mg/g) as the most abundant constituents. Strong antioxidant activity was observed for PCEA in both DPPH (IC_{50} 0.0725±0.0022 mg/mL) and *β*-carotene (45±2.96%) assays. On the other hand, potent anti-inflammatory effect was shown by PCEA with a maximum percentage of 94.52%±0.41compared to the one of diclofenac (59.24 %±1.20).

Keywords: *Pardoglossum cheirifolium*, *Cynoglossum cheirifolium,* HPLC, GC-MS, antioxidant, anti-inflammatory, flavonoids, phenolic acids

1. Introduction

With about 146 genus and 2000 species, the Boraginaceae family can be found almost everywhere around the globe (Gharib and Godarzee, 2016**)**, distributed as annual herbs, shrubs and trees (Botineau, 2010). In folk medicine, leaves, flowers and roots of different Boraginaceae plant species were used to treat injuries, skin diseases, fever, chest pain, joint pain and burn relief (Attard et al., 2012;Ynalvez et al., 2018), in addition to their antibacterial, antiviral, antioxidant and anti-inflammatory effects (Gharib and Godarzee, 2016). The genus *Pardoglossum* is represented by 50 species distributed all over the world, in particular, in the Mediterranean basin with 20 species (Selvi *et al*., 2012).Previous studies reported the antioxidant, antidiabetic, antitumor and anti-inflammatory properties of some *Pardoglossum* species(Joshi et al., 2016). *P. cherifilum* is a plant that grow to 30–40cm, with silky leaves and red corollas(De Lamarck and De candolle, 1815). Previous reports revealed the presence of pyrrolizidine alkaloids, flavonoids and phenolic acids in the plant extracts (Benamar et al., 2021a,b ; Bensaid et al., 2017; 2018). In this paper, we're dealing with the chemical composition, *in vitro* antioxidant and anti-inflammatory properties of both chloroform (PCC) and ethyl acetate (PCEA) extracts from *P.cheirifolium* (L.) Barbier & Mathez.

2. Materials and methods

2.1. Plant material

The aerial parts of *P. cheirifolium* were collected in Spring 2017 from Constantine city (latitude 36°20'26.2"N, longitude 06°37'24.6"E), Eastern Algeria. The plant was authenticated based on botanic books (Quezel and Santa, 1963; Barbier and Mathez, 1973). A voucher specimen has been deposited in the Herbarium of the *VARENBIOMOL* Research Unit, Université des Frères Mentouri, under the flowing order n° 13/2017/PCC.

2.2. Extraction

The aerial parts of the *P. Cheirifolium* (325.30 g) were air-dried at room temperature and then extracted with Soxhlet apparatus using methanol. The methanol extract was concentrated under vacuum at 37°C, to obtain 56.5 g of crude methanolic extract, then diluted with distillated water and subjected to liquid-liquid extraction with chloroform (PCC), ethyl acetate (PCEA) and *n*-butanol (PCB), successively. After evaporation, the extract's weight was 0.38, 0.73 and 12.9 g, respectively. Extracts were stored in closed vessels at 4˚C.

2.3. Metabolite profile by GC-MS analysis of the chloroform extract (PCC)

GC-MS analysis was performed on SHIMADZU GCMS-QP2020. The 70 eV mode of ionization electron impact was used for the GC-MS operation. A 2µL (split ratio of 10:1) injection volume of helium (99.99%), flowing at a steady 1 mL per minute was used as the carrier gas. The temperature of the ion source and the injector was maintained at 280°C and 250°C respectively. The oven temperature was set to increase from 110°C to 200°C at a rate of 10°C per minute ; then to 280°C at a rate of 5°C per minute; with a final 9 minutes hold isothermal at 280°C. The compounds were separated in the column then entered the detector, which creates an electronic signal for each detected compound. The signal was treated by a computer to create a chromatogram. The time from injection to elution of one compound is called retention time (R_t). At 70 eV, the Mass spectral analysis was taken with a 0.5 seconds scan-interval. Total GC and MS running time was 36 min each. A data base was used to elucidate the GC-MS Mass spectrum. The spectrum of the unidentified compounds was compared with known compounds in the database. The retention time, molecular formulae, molecular weight, and peak area with composition percentage of the test plant material were identified.

2.4. Metabolite profile by HPLC analysis of the ethyl acetate extract (PCEA)

The analysis was performed on an HPLC-DAD (1260–Agilent Germany) using a C18 column (Agilent zorbax eclipse ×db size 3µm 4.6 mm × 2.5cm). The mobiles phases A and B were prepared from 0.1% formic acid in water and 100% acetonitrile respectively. The flow rate was set to 0.5 mL/min and the run was maintained at 40% and the injection volume was adjusted to 5µL. Regarding the detection, it was performed in DAD (UV-VIS) at three wavelengths (254, 280 and 330nm). The identification was carried out by extrapolating the chromatograms of the detected compounds to that of the standard molecules and comparing their retention time.

2.5. Antioxidant activity

2.5.1. DPPH radical scavenging essay

The antiradical activity of the two extracts was investigated according to Aree et al.,(2016). DPPH was used as a relatively stable free radical. In this test the antioxidants reduced DPPH with a purple color to a yellow compound; the color intensity is conversely proportional to the capability of antioxidants present in the extracts to produce protons. a volume of 100 µL of

sample solutions was added to 2 mL of DPPH (2.4mg dissolved in 10 mL of methanol). At the same time, a negative control was prepared by mixing 100µL of methanol with 2 mL of DPPH solution. After 30 min incubation in a dark place at room temperature the absorbance was measured against a reference solution at 517 nm. The positive control was represented by a solution of reference antioxidants (ascorbic acid and alpha tocopherol) their absorbance was also measured under the same conditions as the samples, the free radical screening activity was estimated using the equation below:

$$\%DPPH\ Scavenging\ Activity = \frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}} \text{ x } 100$$

Where the $Abs_{control}$ is the absorbance of the control and the Abs_{sample} is the absorbance of the sample

2.5.2. Beta carotene bleaching essay

The β-carotene bleaching activity was evaluated using the β-carotene-linoleic acid system described by Tepe et al.,(2006) with modifications. A solution of 0.5 mL of beta carotene, 200 µL of tween 40 and 25 µL of linolic acid is added to 1 mL of chloroform. After evaporation in vacuum of the chloroform, 50 mL of hydrogen peroxide (H_2O_2) is added under vigorous agitation. The absorbance of the solution is then adjusted to 0.5−0.6 nm. Volumes of 160 µL of this solution are added to 40 µL of samples at different concentrations. The emulsion system was incubated for 2 h at 45°C. Ascorbic acid and α-tocopherol were used as references. The absorbance was measured at 470 nm each 30 min for 2 hours.

The inhibition percentages of β-carotene bleaching were calculated using the following formula:

$$Inhibition\ percentage\ \% = \frac{(\text{D. OE } 120 - \text{D. OT } 120)}{(\text{D. OT } 0 - \text{D. OT } 120)} \text{x} 100$$

Where D.O E_{120} is the absorbance of extract at 120 min, D.O T_{120} the Absorbance of control at 120 min and D.O T_0 is the absorbance of control at 0 min.

2.6. Anti-inflammatory activity

The anti-inflammatory activity of both extracts was evaluated according to the inhibition of thermal protein denaturation modified test described by Karthik et al., (2013). For the test, different concentrations were prepared from 0.0312 to 1mg/mL. 1mL of the sample solutions were added to 1mL of 0.2% ovalbumine (diluted in PBS). The mixtures were heated at 72°C

for 5 minutes and then cooled for 10 minutes. The absorbance of the solutions was measured at 660 nm, against a positive control solution. The anti-inflammatory drug, diclofenac, was used as a reference. The inhibition percentage was determined using the following formula:

$$Denaturation\ Inhibition\ \% = \frac{(\text{absorbance of control} - \text{absorbance of extract})}{\text{absorbance of control}}$$

3. Results and discussion

3.1. GC-MS analysis of chloroform extract (PCC)

By using GC-MS technique, we were able to identify seventeen metabolites from PCC (table 1), most of them were hydrocarbons (92.25%), with 2-methylheptacosane (15.02%), pentacosane (12.12%) and tetracosane (10.59%) as major constituents. The extract was also found to contain some fatty acids (3.64%) and their methyl esters (1.32%). By comparing our findings with the one in literature, we established that only phytol, eicosane have been previously identified in the plant extract (Boussalah, 2020), while the other constituents were rarely reported in *Pardoglossum* species.

Table 1. Data obtained from the GC-MS analysis of the chloroform extract of *P. cheirifolium*

No.	R_t	Compounds	Formula	MW	KI*	Area%
1	15.080	*n*-Tridecanoicacid	$C_{13}H_{26}O_2$	214	1677	3.64
2	17.173	9-Tetradecanoic acid (*Z*), methyl ester	$C_{15}H_{28}O_2$	240	1685	1.13
3	17.392	Phytol	$C_{20}H_{40}O$	296	2045	0.45
4	17.533	Tetradecanoicacidmethyl ester	$C_{15}H_{30}O_2$	242	1727	0.19
5	17.788	*Cis, cis, cis*-7, 10, 13-hexadecatrienal	$C_{16}H_{26}O$	234	1824	1.52
6	18.620	Eicosane	$C_{20}H_{42}$	282	2000	0.65
7	20.162	Heneicosane	$C_{21}H_{44}$	296	2100	2.48
8	21.706	Docosane	$C_{22}H_{46}$	310	2200	5.14
9	23.239	2-Methyldocosane	$C_{23}H_{48}$	324	2300	8.89
10	**24.746**	**Tetracosane**	**$C_{24}H_{50}$**	**338**	**2400**	**10.59**
11	**26.221**	**Pentacosane**	**$C_{25}H_{52}$**	**352**	**2500**	**12.12**
12	27.511	2-Methylpentacosane	$C_{26}H_{54}$	366	2600	5.78
13	27.685	2-Methylhexacosane	$C_{27}H_{56}$	380	2700	10.56
14	**29.336**	**2-Methylheptacosane**	**$C_{28}H_{58}$**	**394**	**2800**	**15.02**
15	30.746	2-Methyloctacosane	$C_{29}H_{60}$	408	2900	0.32
16	31.261	2-Methylnonacosane	$C_{30}H_{62}$	422	3000	6.69
17	33.574	Hentriacontane	$C_{31}H_{64}$	436	3100	6.41
		Hydrocarbons				**92.25**
		Methyl esters				1.32
		Fattyacids				3.64
		Total				**97.21**

*Experimental KIs on a DB-5 MS apolar column

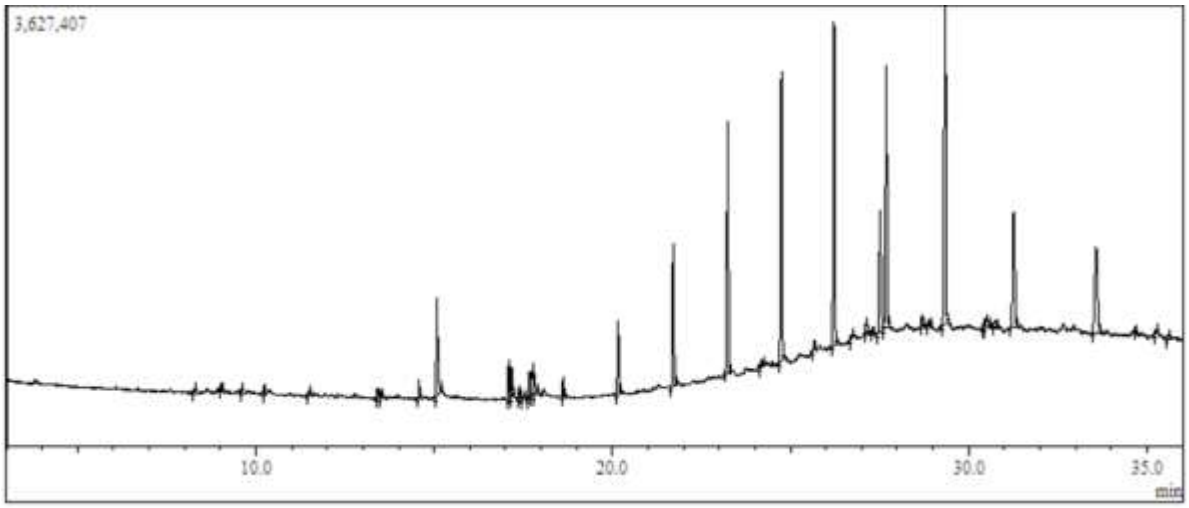

Figure 01: GC-MS chromatogram of the chloroform extract from *P. cheirifolium*

3.2. HPLC analysis of ethyl acetate extract (PCEA)

The examination of PCEA by HPLC, using standard references, allowed the quantification of nine flavonoids (127.548 mg/g) and eight phenolic acids (52.5095 mg/g). The obtained data (table 2) showed that *epi*catechin (106.5625 mg/g) was the most abundant compound in the extract, followed by 2-hydroxycinnamic acid (46.687 mg/g) and catechin (14.393 mg/g). The flavonoids luteolin, naringenin, apigenin 7-glucoside, luteolin7-glucoside, quercetin, rutin and apigenin were also detected but in low concentration. By comparing our findings with the ones in the literature, we conducted that this is the first identification of 3,4-dihydroxybenzoic acid, apeginin-7-glucoside, 2-hydroxycinnamic acid, luteolin and apigenin in the genus *Pardoglossum*.

Table 2. Identification and quantification of the ethyl acetate extract of *P. cheirifolium* the HPLC profile by using standard references

No.	R_t	Compound	mg/mL	mg/g	λ nm
1	7.118	Gallicacid	0.0010	0.1	280
2	10.238	3 ;4-dihydroxybenzoic acid	0.0113	1.13	280
3	11.674	Chlorogenicacid	0.0460	2.875	280
4	**12.092**	**Catechin**	**0.2303**	**14.393**	**280**
5	12.836	Caffeicacid	0.0152	0.95	280
6	**13.340**	***Epi*catechin**	**1.7051**	**106.5625**	**280**
7	14.202	Vannilicacid	0.0019	0.118	254
8	15.255	Rutin	0.0002	0.0125	254
9	16.607	Luteolin7-glucoside	0.0052	0.325	330
10	17.008	*p*-Coumaricacid	0.0006	0.0375	330
11	18.276	Apeginin-7-glucoside	0.0083	0.518	330
12	18.274	Ferrulicacid	0.0098	0.612	280
13	**18.908**	**2-hydroxycinnamicacid**	**0.747**	**46.687**	**280**
14	21.20	Naringinin	0.0162	1.012	280
15	23.249	Querctetin	0.0012	0.075	254

16	23.335	Luteolin	0.074	4.625	330
17	26.011	Apeginin	0.0004	0.025	280
		Phenolicacids	0.9328	52.5095	
		flavonoids	2.0409	127.548	
		Total	2.9737	180.06	

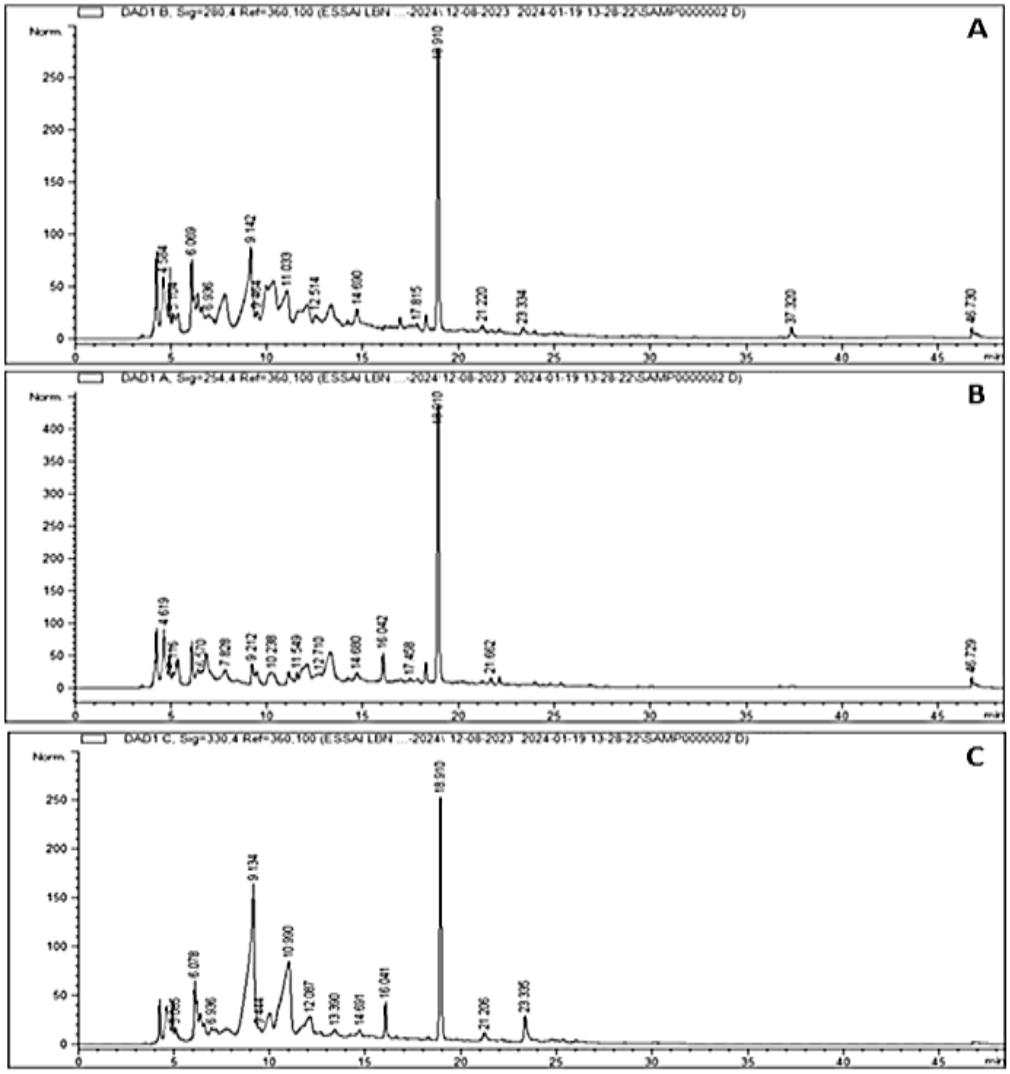

Figure 02: HPLC chromatograms of PC ethyl acetate extract A: at 254 nm, B: at 280 nm, C: at 330nm

3.3. Antioxidant activity

In the aim of determining the antioxidant potential of the studied plant extracts, PCC and PCEA were subjected to *in vitro* DPPH free radical-scavenging and *β*-carotene/linoleic acid assays. Results (table 3) showed that PCEA exert strong DPPH free radical-scavenging activity with an IC_{50} values of 0.0725±0.0022 mg/mL, similar to the one obtained by ascorbic acid (0.039±0.022 mg/mL), while PCC showed a very similar IC_{50} value (0.166±0.032

mg/mL) to the one of α-tocopherol (0.146±0.030 mg/mL). On the other hand, *β*-carotene/linoleic acid confirmed that PCEA exhibit stronger antioxidant activity than PCC, where the IC_{50} value of the first one (45±2.96 mg/mL) was quite similar to the obtained ones for both ascorbic acid (43.54±0.91 mg/mL) and α-tocopherol (46.93±1.14), while the effect of PCC (28.70±1.82 mg/mL) was inferior than both standards and PCEA. Our findings can be related to the chemical composition of PCEA, rich in flavonoids (127.548 mg/g) and phenolic acids (52.5095 mg/g), in particular, *epi*catechin (106.5625 mg/g), 2-hydroxycinnamic acid (46.687 mg/g) and catechin (14.393 mg/g), reported previously to exert potent antioxidant properties (Maurya and Devasagayam 2010; Anitha et al., 2021; Jug et al., 2021).

Table3: *In vitro* antioxidant activity of *P. cheirifolium* extracts

	DPPH assay IC_{50}(mg/mL)	*β*-carotene assay I% (mg/mL)
PCEA	0.0725±0.0022	45±2.96
PCC	0.166±0.032	28.70±1.82
Ascorbic acid	0.039±0.022	43.54±0.91
α-tocopherol	0.146±0.030	46.93±1.14

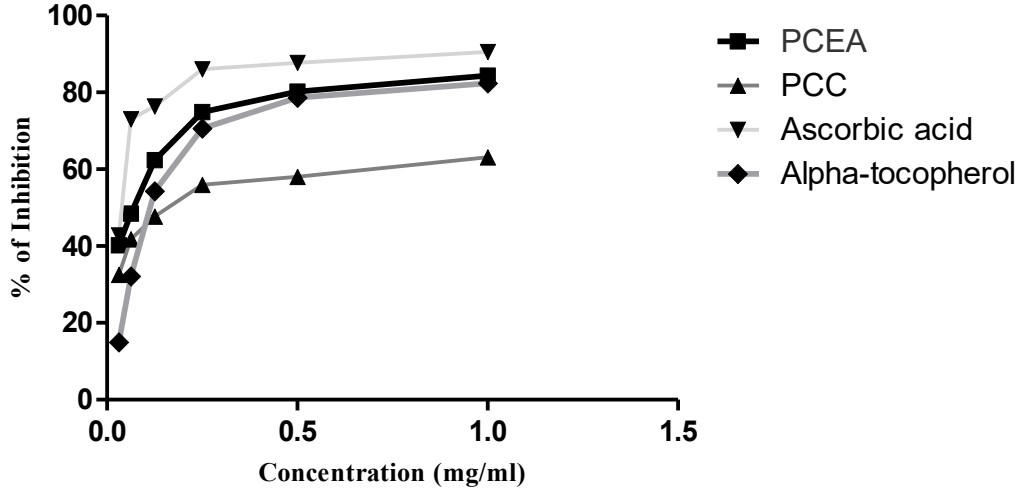

Figure 03: % DPPH scavanging activity of *P. cheirifolium* extracts

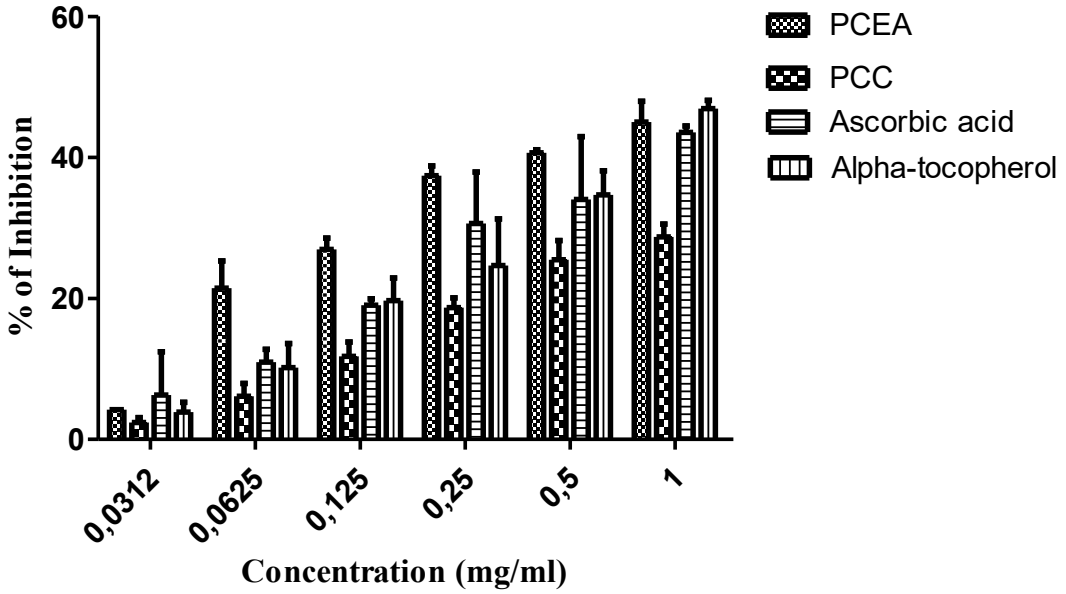

Figure 04: *β*-carotene Inhibition percentage of *P. cheirifolium*extracts

3.4. Anti-inflammatory activity

The results obtained from the inhibition of thermal protein denaturation test showed a very significant anti-inflammatory potenial for PCEA (94.52%±0.41) and PCC (79.79% ±1.47) compared to the reference drug diclofenac (59.24 %±1.20). This findig indicates that *P. cheirifolium* extracts, in particular PCEA, exerts a very potent anti-inflammatory inhibition even in lowest dose (0.0312 mg/mL) with an inhibition percentage of 85.29%±0.30. The potent activity of PCEA may be associated to the presence of good amount of flavonoids and phenolic acids, where many studies indicated that plant polyphenols inhibit different pro-inflammatory mediators in both *in vitro* and *in vivo* models (Sangiovanni et al., 2020). For example, Nile et al., (2015) reported the promising anti-inflammatory activity of some phenolic acids, in particular, gallic acid, ferulic acid, caffeic acid and *p*-coumaric acid, while the study by Chunlian et al., (2021) demonstrated how luteolin, apigenin and quercetin exhibits remarkable anti-inflammatory properties. In addition to phenolic compounds, the plant known for containing pyrrolizidine alkaloids (benamar et al., 2021a,b). This kind of secondary metabolites were reported for their anti-inflammatory capacities (Aboelmagd et al., 2018).

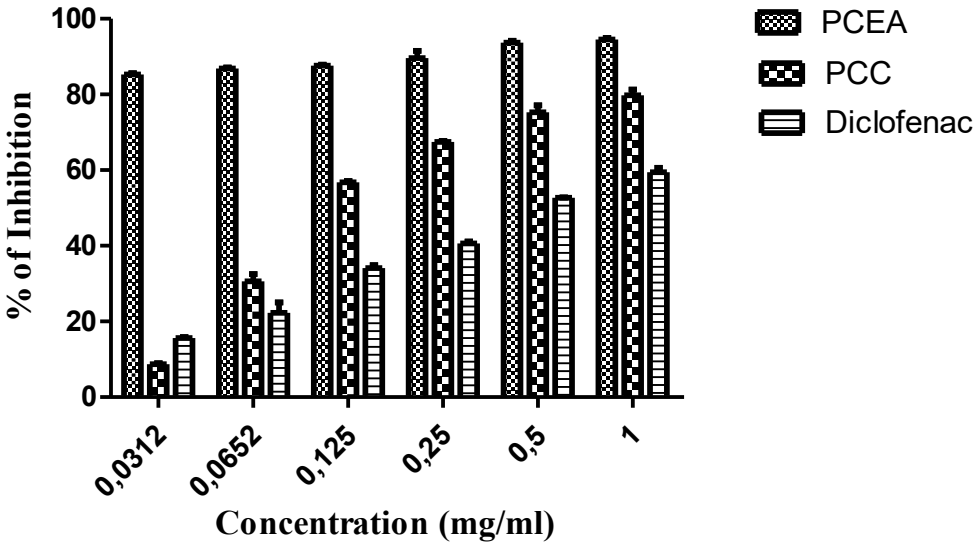

Figure 05: Anti-inflammatory inhibition percentage of *P. cheirifolium*extracts

Conclusion

This work was aimed to enhance the available information's on *Pardoglossum cheirifolium* by examining its chemical composition, *in vitro* antioxidant and anti-inflammatory properties of both chloroform and ethyl acetate extracts. Nine flavonoids and eight phenolic acids were identified, supporting the potent anti-inflammatory and anti-oxidant activities of both extracts. More studies are needed to understand the potent biological properties.

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