Original Paper

Preliminary Study on the Antioxidant Effect of Natural Based Products with Potential Application in Complex Regional Pain Syndrome

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ABSTRACT: Complex regional pain syndrome (CRPS) is a complex condition characterized by chronic pain and various sensory, motor, and autonomic symptoms. It involves a complex interplay of mechanisms in the nervous system, including neuroinflammation, sensitization of pain pathways, and dysfunction of the sympathetic nervous system. Antioxidants may play a role in CRPS by helping to counteract oxidative stress, which is an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defences. CRPS involves inflammation and tissue damage, which can lead to increased ROS production and oxidative stress. Our paper represents a preliminary study on various commercially available natural-based products regarding their antioxidant effect. Several natural products with antioxidant properties, such as vitamins C and E, polyphenols, flavonoids, and botanical extracts, have shown promise in preclinical studies for their potential to alleviate pain and reduce inflammation associated with CRPS. The potential use of natural-based products with antioxidant effects for mitigating CRPS symptoms is still an area of ongoing research and investigation, but nonetheless it holds promise.

KEYWORDS: *CRPS, antioxidant, DPPH, natural products.*

Introduction

Complex regional pain syndrome (CRPS) is the current name for the syndrome formerly known as reflex sympathetic dystrophy or causalgia [1].

In addition to the classic features of neuropathic pain-intense burning, hyperalgesia and allodynia, CRPS is associated with local edema and changes that suggest autonomic involvement-hypersweating, changes in the colour and temperature of the skin of the affected region.

Trophic changes in the skin, hair and nails can also occur, as well as alteration of motor function-loss of muscle strength, decreased active range of motion and tremors [2].

CRPS is a chronic, debilitating, painful condition that is relatively poorly understood from a pathophysiological point of view and for which there are no specific laboratory tests, the diagnosis of CRPS being established exclusively by clinical criteria.

Thus, an early diagnosis and multidisciplinary treatment is needed to prevent the onset of muscle atrophy and motor deficit.

Also, the psycho-emotional impact can maintain the vicious circle of clinical manifestations and affect the quality of life of patients.

CRPS poses significant challenges when it comes to chronic pain management [3].

Despite its prevalence, there is currently no established pharmaceutical treatment available, and clinical trials have yielded insufficient evidence to support the effectiveness of commonly employed interventions [4,5].

Consequently, costly and invasive palliative measures like spinal cord stimulation and intrathecal drug delivery systems are frequently employed.

The limited success in finding suitable treatments for CRPS can be attributed to a lack of comprehensive understanding regarding its underlying pathophysiological mechanisms.

The pathophysiology of CRPS I is influenced by the production of free radicals through the mitochondrial respiratory chain.

Patients with CRPS exhibit notable elevated levels in malondialdehyde, lactic dehydrogenase, and antioxidants, as evidenced by studies conducted on their serum and particularly their saliva, when compared to healthy individuals [6].

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Moreover, Coderre *et al*. observed elevated malondialdehyde concentrations in the muscles of rat hind paws.

They also provided evidence that pain hypersensitivity in animals can be mitigated through the use of free radical scavengers and antioxidant treatment. [7].

Given that mitochondria are the primary origin of reactive oxygen species (ROS), it is plausible to propose that oxidative stress-induced mitochondrial dysfunction might contribute to the development of CRPS [8].

Furthermore, mitochondrial dysfunction has been acknowledged as a significant instigator of various disorders, such as neurodegeneration, diabetes, and ischemia-reperfusion injury [9,10].

Consequently, the pursuit of mechanism-based treatment has been a longstanding objective in the management of CRPS, and advancing our understanding of its pathophysiology holds the potential to realize this goal.

By comprehending the pathways through which oxidative stress impacts CRPS, we can gain deeper insights into the biological mechanisms underlying its development, which in turn may pave the way for the identification of enhanced therapeutic approaches [11].

For centuries, natural products have been utilized in medicine, and their potential benefits in CRPS extend beyond addressing the pathophysiological aspects of the disease.

They also show promise in alleviating associated comorbid conditions.

Curcumin, ginger, nutmeg, cinnamon, white willow bark, and boswellia are some of the products known for their anti-inflammatory properties [12].

In our study we aimed to assess the antioxidant effect of several products that are commonly found on the market, and which can further be used as adjuvants in the management of CRPS.

We chose products that contained extracts only from *Boswellia serrata*, *Curcuma longa* and *Harpagophytum procumbens* or mixtures between them or other extracts.

Material and Methods

Chemicals and solvents

All HPLC (LiChrosolv®) gradient grade solvents (methanol, water, chloroform) were purchased from Merck (Darmstadt, Germany).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Aldrich (Taufkirchen, Germany).

Sample preparation

We purchased four of the most common products marketed for rheumatological use. The products' main components were:

1. *Curcuma longa*/*Boswellia serrata*/*Zingiber officinale* (**S1**);
2. *Curcuma longa*/Piperine (**S2**);
3. *Harpagophytum procumbens* (**S3**);
4. *Boswellia serrata* (**S4**).

The working samples were obtained by extracting 1g of product with 10mL solvent (70% methanol).

The extraction process consisted of ultrasonication the mixture at 120W and 50℃ for 15 minutes using a Bandelin Sonorex DL102H ultrasound bath (Bandelin Electronic GmbH&Co. KG, Berlin, Germany).

After ultrasonication the samples were centrifuged at 10 000 RPM using an Eppendorf 5804 centrifuge (Eppendorf, Hamburg, Germany) for 15 minutes.

All samples were filtered through 0.22µm syringe filters (Acrodisc MS Syringe Filters WWPTFE Membrane-Fisher Scientific, Göteborg, Sweden) prior to application.

DPPH Assay

The radical scavenging ability against DPPH radical was measured by the means of the IC_{50}.

The IC_{50} (half maximal inhibitory concentration) represents the concentration of a compound or sample that reduces the DPPH radical scavenging activity by 50%.

To determine the IC_{50} value for DPPH a series of dilutions of the samples, covering a range of concentrations, were performed (100mg/mL to 49µg/mL).

In a 96-well plate, 50µL of each sample was combined with 150µL of a 200µM methanolic solution of DPPH.

All samples were tested in triplicate.

The plate was then left in the dark for 20 minutes, after which the absorbance was measured at 520nm using a BMG FLUOstar Optima (Ortenberg, Germany) plate reader [13].

The raw data was statistically analyzed using the GraphPad Prism 8.0 Software.

The IC_{50} value was obtained by plotting log(inhibitor) vs. normalized response.

HPTLC-UV/Vis/FLD-EDA

Sample application on the plates was carried out using a Linomat 5 instrument from CAMAG (Muttenz, Switzerland), while densitometry was performed using a TLC Scanner 3, also from CAMAG.

Both instruments were controlled using the CAMAG visionCATS v2.5 software package.

The derivatization process was conducted using the CAMAG Chromatogram Immersion Device.

A twin-trough glass chamber (20cm×10cm) was used for plate development.

The resulting plate was documented using a Canon 700D digital camera (Tokyo, Japan).

HPTLC Si 60 F_{254} 20cm×10cm glass plate was used.

Samples were applied on the glass plate using the Linomat 5 as follows: band length 8mm, distance from the lower edge 8mm, dosage speed 150nL/s, application volumes 2µL/band.

The development was performed with chloroform: methanol (90:10 *v*/*v*). Documentation was performed at 254nm (UV), 366nm (FLD) and in white light for DPPH.

The HPTLC-EDA assay was conducted by submerging the glass plate after elution for one second in 0.04% methanolic DPPH.

Yellow bands on a purple background were generated instantly [14,15].

Statistical analysis

The IC_{50} value of each sample was determined using a nonlinear regression of the logarithmic concentration versus the normalized response of the antioxidant agent [16].

The data are presented as the mean and standard deviation.

A two-way ANOVA test was used to examine the differences between samples.

A p value less than 0.05 was considered statistically significant.

The nonlinear regression and statistical analyses were performed using the GraphPad Prism 8 software (San Diego, CA, USA).

Results

The competitive and/or synergistic effects of individual compounds can occur within complex mixtures of compounds such as plant extracts.

To assess the overall impact of bioactivity, total parameter assays are a great initial tool to be used.

However, it is crucial to investigate the individual active compounds to ensure the effectiveness of the botanicals and identify the nature of the active ingredient, whether it's a phytochemical, xenogenic contaminant, or residue.

Gathering information through bioactivity profiling is a necessary prerequisite for quality control of potent botanicals.

In addition to the microchemical analysis, which revealed the presence of flavonoids as yellowish fluorescent zones under UV 366nm illumination (Figure 2), effect-directed detections can also be utilized.

The radical scavenging properties of individual compounds within the separated plant extracts were assessed by immersing them in the purple DPPH reagent.

Radical scavengers were identified as bright yellow zones against a purple background on the HPTLC plate under white light illumination (Figure 3).

Over a 24-hour observation period, the yellowish 2,2-diphenyl-1-picrylhydrazine zone increased in intensity.

Despite the interference caused by its yellow colour, the curcumin zone exhibited remarkably strong radical scavenging properties, which were significantly higher compared to the individual bioactive components found in the extracts of *Boswellia serrata* and *Harpagophytum procumbens* (using the same amount and volume of sample).

DPPH Assay

Using 8 to 13 points (n=3) per curve for each nonlinear analysis we obtained accurate results for the DPPH IC_{50} assay which are presented in Table 1.

A lower IC_{50} value indicates a more potent compound, as it requires a lower concentration to achieve the desired effect.

The products containing curcumin showed significantly lower values than the other two, thus presenting a more intense antioxidant effect (p<0.05).

Table 1. DPPH IC50 values for all analysed samples.

Sample	IC_{50} [mg/mL]	R^2
S1	0.093±0.012	0.9851
S2	0.383±0.049	0.9871
S3	3.382±0.715	0.9690
S4	22.19±5.580	0.9306

HPTLC-UV/Vis/FLD-EDA

For two of the samples (S3,S4) multiple higher volumes were used to obtain a better signal for the eluted compounds.

Under the current chromatographic conditions only three (S1,S2,S4) of the four samples managed to be separated.

For the first two samples curcumin is observed at an R_F of 0.58 while for the second sample (S2) piperine is also found at an R_F 0.77.

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A lower intensity in curcumin is observed for the first sample (S1) probably due to the fact that multiple active extracts were used in its composition.

No separation of compounds was observed for the third sample (S3).

For the fourth sample (S4) boswellic acid derivatives are present at an R_F of 0.45 and 0.58, respectively (Figure 1, 2).

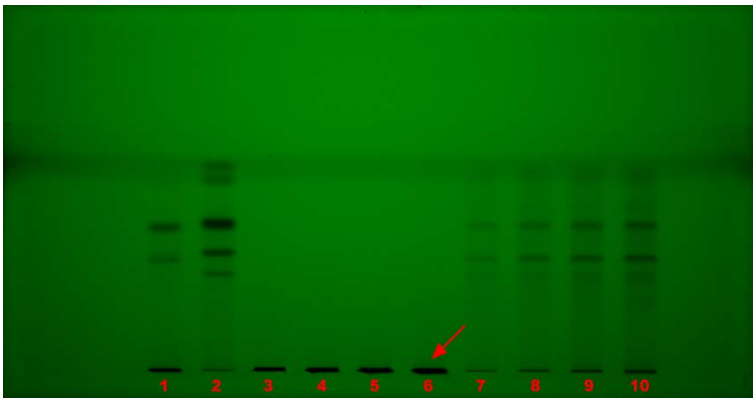

Figure 1. HPTLC profiling of the four samples at 254 nm (1-S1; 2-S2; 3-6-S3; 7-10-S4).

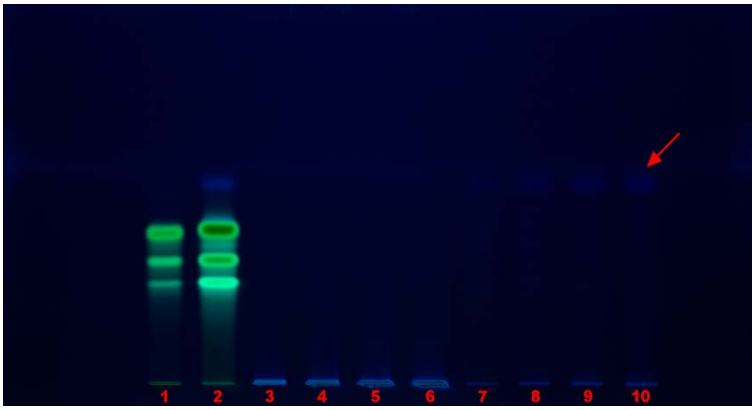

Figure 2. HPTLC profiling of the four samples at 365nm (1-S1; 2-S2; 3-6-S3; 7-10-S4).

The DPPH derivatization of the plate revealed information in accordance with the IC_{50} values obtained previously.

Evident yellow zones are present in the first two samples with higher intensity in the second sample.

For the first sample (S1) multiple bands are present at the R_F above curcumin which are not present either at 254nm or at 365nm.

High intensity yellow zones are observed in the start area for both the third (S3) and the fourth sample (S4).

In the last sample hardly, visible yellow zones are present at an R_F 0.66 (Figure 3).

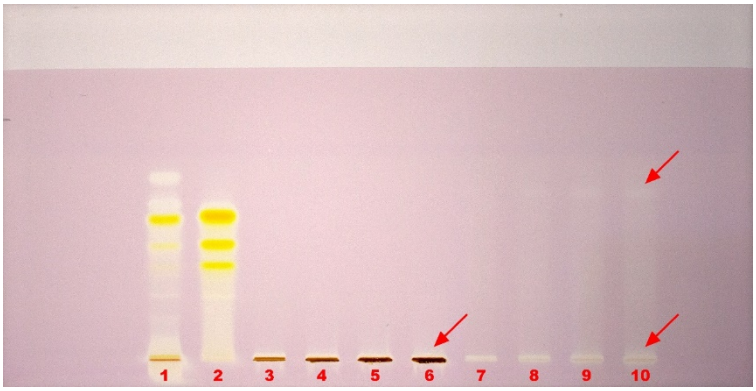

Figure 3. HPTLC antioxidant profiling of the four samples in white light derivatized with DPPH (1-S1; 2-S2; 3-6-S3; 7-10-S4).

Discussion

Complex regional pain syndrome (CRPS) is a chronic pain condition characterized by persistent and severe pain, often accompanied by sensory and autonomic disturbances.

The exact cause of CRPS is not fully understood, but it is believed to involve abnormal inflammation and dysfunction in the peripheral and central nervous systems.

They interact and maintain a vicious circle of events, the result of which is persistent pain, which is accompanied by vasomotor, sudomotor and trophic changes.

Mechanisms such as inflammatory changes and impaired microcirculation, which can contribute to oxidative stress and immune dysfunction, have been suggested as significant factors in tissue damage.

Oxidative stress arises from an imbalance between the generation of reactive oxygen species (ROS) and the body's capacity to counteract and eliminate them with antioxidants. ROS, which are exceptionally reactive molecules, have the potential to harm cells and play a role in numerous diseases and the aging process. [17].

Under normal circumstances, the body produces ROS as byproducts of cellular metabolism, and antioxidants help neutralize them.

However, when there is an excess production of ROS or a deficiency in antioxidants, oxidative stress can occur.

This can be caused by various factors, including environmental pollutants, toxins, certain medications, chronic inflammation, and unhealthy lifestyle habits such as poor diet, smoking, and excessive alcohol consumption [18].

Oxidative stress can damage cellular components, including lipids, proteins, and DNA, leading to cellular dysfunction, and potentially contributing to the development of various diseases such as cardiovascular diseases, neurodegenerative disorders (e.g., Alzheimer's and Parkinson's disease), cancer, diabetes, and chronic inflammatory conditions.

While some levels of oxidative stress are a natural part of cellular metabolism, excessive or prolonged oxidative stress can be detrimental to health.

Therefore, maintaining a balance between ROS production and antioxidant defences is crucial for optimal cellular function and overall well-being [19].

In CRPS the hypoxia is caused by either extreme vasoconstriction, excessive sympathetic activity or is the result of a local imbalance between endothelial factors.

Consequently, a reduction in oxygenation of the capillaries was observed, while dermal microdialysis revealed elevated lactate levels [20].

Additionally, nuclear magnetic resonance spectroscopy of the muscles unveiled indications of acidosis and a decline in phosphate metabolism.

Consequently, the affected segments exhibit histopathological features that align with oxidative stress.

Hypoxia induces acidosis and the generation of free radicals, which in turn trigger severe pain sensations in the associated nerve fibers [21].

The presence of free radicals can amplify inflammation, increase vascular permeability, release neuropeptides, and lead to tissue damage.

There is evidence suggesting that antioxidants, such as free radical scavengers like N-acetylcysteine, dimethylsulfoxide, mannitol, and vitamins C or E, as well as coenzyme Q10, may hold promise in alleviating symptoms associated with CRPS.

These antioxidants can help reduce oxidative stress and inflammation, which may alleviate pain and improve overall the quality of life in individuals with CRPS [22].

After assessing the IC_{50} values of all the samples we found that the products containing curcumin extracts had the lowest values indicating a high antioxidant effect.

Curcumin is a polyphenolic compound which is commonly found in the *Curcuma* spp. and it is also known for its inhibition to prevent the release of pro-inflammatory mediators.

Furthermore, these compounds exhibit an exceptional absence of harmful effects, as evidenced by the fact that the *Curcuma* rhizome is approved for human ingestion and its widespread application as a culinary seasoning [23,24,25,26].

The product which contained *Harpagophytum procumbens* or commonly known as devil's claw showed a higher IC_{50} value than the curcumin products.

Though, we couldn't separate the compounds from the extract (most likely because the harpagosides are polar compounds and did not elute in our nonpolar mobile phase) we can still observe the evident yellow intensity of the extract mixture at the application point on the plate [27,28].

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The least antioxidant effect was observed in the product which contained *Boswellia serrata*, although there are some feint yellow zones in the separated profile (mostly in the application zone).

Boswellic acids are primarily recognized for their anti-inflammatory properties.

Studies have demonstrated that *in vitro*, 11-keto-boswellic acids can effectively inhibit 5-lipoxygenase, which is a crucial enzyme involved in leukotriene biosynthesis.

This inhibition could be responsible for their anti-inflammatory effectiveness [29,30].

However, it is important to note that research on the use of products based on natural extracts for CRPS is limited, and the available evidence is not conclusive.

Antioxidants are substances that can help protect cells from damage caused by harmful molecules called free radicals.

Their use in the management of CRPS has been explored as a potential approach to address oxidative stress and inflammation, which may play a role in the development and progression of the condition.

However, it is important to note that the evidence regarding the specific use of antioxidants for CRPS is limited, and further research is needed to establish their effectiveness [31].

While the results of studies are promising, it is important to note that larger, well-designed clinical trials are needed to establish the effectiveness and optimal dosages of antioxidants in CRPS management.

It is worth mentioning that a comprehensive approach to CRPS management typically involves a combination of interventions, including physical therapy, medications, nerve blocks, psychological support, and lifestyle modifications.

Antioxidants may be considered as part of a multimodal treatment plan, but their use should be guided by medical advice and in conjunction with other appropriate therapies.

Conclusions

CRPS is a complex pain condition, and its treatment typically focuses on managing symptoms, reducing inflammation, improving circulation, and addressing the underlying causes.

While antioxidants play a role in overall health and may have potential benefits in managing inflammation, it is important to note that CRPS is a multifaceted condition that requires a comprehensive approach to treatment.

Our study showed that products that are readily available on the market and used for rheumatological conditions proved their antioxidant effectiveness and could be adopted as adjuvants in reducing the oxidative stress in CRPS.

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Conflict of interests

None to declare

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