PhD Theses

Ultrasonic Extraction of Bioactive Compounds from *Cannabis Sativa* L. for the Green Reduction of Graphene Oxide on Cellulose Fibres

by

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under the supervision of

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in

Material Science and Technology

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Problem area

Nanomaterials have become an inseparable part in the present times, playing a dominant role in various sectors such as biomedicine, catalysis and biosensing. The two major pathways for the synthesis of nanomaterials are the bottom-up and top down approaches. Both approaches rely on physico-chemical methods, which employ chemical agents for the reduction and stabilization of the nanomaterials. Most of these chemical agents are toxic or corrosive in nature and lead to hazardous by-products. This is especially undesirable in case of biomedical healthcare and diagnostic applications. Moreover, they require an energy-intensive reaction environment and are expensive. Thus, there is an intense need for eco-friendly agents for the synthesis of nanomaterials having non-toxic reaction products [1, 2].

In view of this, a number of eco-friendly agents have garnered the attention of the scientific community, which include entities of biological origin such as microorganisms, enzymes, plant extracts, etc. Of these, the plant extracts are particularly advantageous due to their wide availability and cost-effectiveness. Plant extracts have proven potential to act not only as natural reducing agents for the synthesis of nanomaterials but also as capping agents for the stabilization of the synthesized nanomaterials. They need a much less energy-intensive environment and can react in ambient conditions of temperature and pressure. Further, they can be used to synthesize nanomaterials in large quantities free of chemical contaminants and hence, hold potential for industrial scale-up [3, 4].

Lately, non-conventional techniques have been lately focussed on which include extractions assisted by ultrasound, microwave, enzymes, pulsed electric field, supercritical fluid extraction and pressurized liquid extraction. Ultrasonication has been widely employed for the extraction of bioactive compounds owing to several advantages like shorter extraction time and small amount of solvent requirement. The extract thus obtained is rich in natural reducing compounds that can be exploited to achieve facile reduction and synthesis of a variety of nanomaterials [5-7].

Objectives

In light of the aforementioned problem, the following research objectives were proposed:

- ✓ Extraction of bioactive compounds (cannabinoids, terpenes, flavonoids, etc.) from the inflorescence of *Cannabis sativa* L. using ultrasonication at varying experimental conditions
- ✓ Evaluation of the extracts to obtain the response values (total phenolics, total flavonoids, ferric reducing ability of plasma and extraction yield)
- ✓ Optimization of the extraction parameters (time, ultrasonic power and extraction solvent) by response surface methodology
- ✓ Qualitative analysis of the extract using chromatographic techniques (HPLC-DAD-MS/MS and GC-MS)
- ✓ Utilization of the extract for in situ reduction of graphene oxide (GO) on cellulose fibres
- ✓ Characterization of the reduced-GO/cellulose composites using advanced analytical techniques (FTIR, SEM, XRD and XPS) and study of their electrical performance

Research methodology

Extraction:

Plant sample (2.5 g) were mixed with 50 ml of solvent (20, 50 or 80% methanol) and extracted at 90, 120 or 150 W power, keeping the time of sonication at 5, 10 and 15 min. Subsequently, the extracts were cooled, filtered and stored away from light. A control extraction was performed for comparative study by mixing the plant sample with

50% methanol with continuous stirring at 300 rpm and 60 °C for 30 min on a magnetic stirrer, followed by cooling and filtering.

Extract evaluation/response determination:

The prepared extracts were evaluated for their total phenolic content (TPC), total flavonoids (TF), ferric reducing ability of plasma (FRAP) and the extraction yield using standard protocols.

Optimization of parameters/experimental design:

Face-centered central composite design (CCD) was used to investigate the effect of different input variables on the studied output parameters and to optimize the extraction process. For a 3-factor CCD, 20 experimental runs with various combinations of the factors and comprising of 8 factorial points (coded as ±1), 6 star or axial points (coded as ±α) along with 6 replicates of the centre point (coded as 0) were performed randomly. Using Design Expert Software, the analysis of variance (ANOVA) for each of the responses was performed with a 95% confidence interval to determine significant differences within means based on the probability or 'p-value' ($p < 0.05$). The optimal parameters were obtained by maximising the desirability function (D).

Analysis of the Cannabis extract:

The methanolic extracts of *Cannabis* were analysed for cannabinoids and other bioactive constituents using high pressure liquid chromatography/mass spectrometry (HPLC-DAD-MS/MS) and gas chromatography/mass spectrometry (GC-MS) techniques, respectively.

Green reduction of GO on cellulose fibres:

The *Cannabis* extract was used as a natural reducing agent for the green reduction and simultaneous functionalization of GO on cellulose fibres without external stabilizers. GO suspensions were made by dispersing the GO powder (0.05 g) in deionized water (70 ml) followed by sonication for 15 min. The cellulose pulp was prepared by disintegrating the linter sheets in a Lorentzen and Wettre pulp disintegrator at 1% consistency for 10 min and then taking a volume corresponding to 5 g of air-dried fibres. Further, the sonicated suspensions were treated with the extract (10 ml) in the optimized ratio of about 0.2 ml extract/mg GO, followed by heating to 80 °C for 1 h with occasional stirring. Next, the partially reduced GO suspensions were again sonicated for another 15 min and subsequently poured into the cellulose pulps (500 ml) already preheated to 75-80 °C in different volumes corresponding to different weight fractions of GO viz. 0.1, 1, 2, 5 and 10 m/m %. After 2 h, the pulp mixtures were removed and allowed to cool naturally. Finally, handsheets were made from the treated pulps in a semi-automated sheet machine (HAAGE D-4330 System Laboratory sheet former) with vacuum press-drying (9.0 × 104 Pa, 90 °C) using the DIN EN ISO 5269-2 standard test method.

Analytical techniques such as Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS) were used for the characterization of the prepared reduced graphene oxide (RGO)/cellulose composites. The XRD and XPS measurements were performed on Indus-2 synchrotron source at RRCAT, India.

The surface resistivity of the composites was measured using Keithley 6517B electrometer and Keithley 8009 resistivity text fixture by sourcing a known voltage for 60 s.

Results

Extraction, evaluation of responses & optimization of process:

The influence of three independent factors (time, power and methanol concentration) was evaluated on the extraction of total phenols, flavonoids, FRAP antioxidant assay and the overall yield. Both the solvent composition and the time significantly affected the extraction while the sonication power had no significant impact on the responses. The experimental data for each response was fitted to a second order polynomial equation. The two-factor interaction model well fitted TPC while linear models fitted the other responses of TF, FRAP and yield**.** The process conditions were optimized as: **15 min time**, **130 W power** and **80% methanol**. The response predictions obtained at optimum extraction conditions were 314.822 *mg GAE/g DW* of TPC, 28.173 *mg QE/g DW* of TF, 18.79 *mM AAE/g DW* of FRAP and 10.86% yield. A good correlation was observed between the predicted and experimental values of the responses, thus validating the mathematical model. On comparing the ultrasonic process with the control extraction, appreciably higher values were obtained for each of the responses. Additionally, ultrasound was found to considerably improve the extraction of cannabinoids present in *Cannabis*.

Identification of Cannabis constituents:

Several cannabinoids including THC, CBD, CBGA, CBDA, THCVA, CBLA, CBNA, CBCA, etc. were identified in the extract using HPLC-DAD-MS/MS. The other bioactive compounds identified using GC-MS included mostly the common terpenes, phytols, fatty acids, etc. The possible entourage effects between the cannabinoids and the non-cannabinoid bioactive compounds such as terpenes play a crucial role in determining the pharmacokinetics of the main cannabinoid and thus its overall therapeutic potential.

In situ reduction of graphene oxide on cellulose by Cannabis extract:

Morphological analysis revealed a homogeneous coating of RGO over the fibre surface with uniform dispersion throughout the porous cellulose matrix thus eliminating the need for additional stabilizing agents. The spectroscopic and diffraction techniques confirmed successful removal of oxygen-containing functional groups and in situ reduction of GO on the cellulose fibres. The RGO/cellulose composites exhibited a drop in surface resistivity with increasing RGO content from to $1.81 \times 10^{11}\ \Omega$ for 0.1 m/m % RGO to $0.15 \times 10^{11}\ \Omega$ for 10 m/m % RGO loading at 40 V. Similarly, the surface charging capacity of the composites at 40 V dropped from 1.21×10^{-3} ΔmAh for 0.1 m/m % RGO to 0.05×10^{-3} ΔmAh for 10 m/m % RGO content, which can be exploited for many electrical applications. The presence of air voids within the cellulose fibre matrix significantly affected the electrical properties of the composites. The porosity of the cellulose fibre matrix and the strong interaction between RGO and cellulose played an instrumental role in determining the performance of the composites. Thus, employing *Cannabis* extract for the green reduction of GO seems to be an appealing choice as compared to the conventional reducing agents

Main conclusions of the research

The research work has been summarized in the following bullet points that indicate the achievements as well as implications.

- Extraction of bioactive compounds such as terpenes, flavonoids and cannabinoids was successfully accomplished using the technique of ultrasonication from the inflorescences of *Cannabis*. The extraction was done by varying the ultrasonic parameters of time, power and solvent. (**CA-JA1**)
- The optimization of the extraction parameters was done using a 3-factor central composite design approach. The responses were analysed by fitting a second order polynomial; the TPC was well described by the factor interaction model while linear models described the TF, FRAP and yield. The regression and graphical analysis revealed the solvent composition and time to be the most predominant factors influencing the extraction process, except in case of the FRAP assay. (**CA-JA1**)
- The time had a positive effect on the responses. More methanol content in the solvent favoured the TPC while it negatively affected TF and the extraction yield. The ultrasonic power, on the other hand, did not have any significant impact on any of the responses investigated. (**CA-JA1**)
- Considerably higher values of all the responses were obtained for the ultrasonic extraction than the control one. Ultrasonication also considerably enhanced the extraction of cannabinoids, which was confirmed by HPLC chromatograms. Several cannabinoids including THC, CBD, CBGA, CBDA, THCVA, CBLA, CBNA, CBCA, etc. were identified in the extract using HPLC-DAD-MS/MS. (**CA-JA1, CA-JA3**)

- In situ green reduction of GO with simultaneous functionalization on the cellulose fibres using the aqueous extract from the inflorescences of *Cannabis* was achieved. The successful removal of oxygen-containing functional groups and anchorage to the cellulose fibres was confirmed by spectroscopic and diffraction techniques. (**CA-JA4**)
- The cellulose fibres served a dual role of a supporting matrix with excellent anchorage to RGO resulting from strong electrostatic surface interactions as well as a reductant due to the presence of free hydroxyl and ether groups. (**CA-JA4**)
- The surface resistivity of the composites dropped markedly (by over a 100 orders of magnitude) by incorporation of the conducting RGO in the cellulose fibres. However, it was found to be significantly affected by the presence of air voids, which acted as an obstacle preventing the formation of an effective conductive network of RGO layers within the cellulose matrix. The porosity of the cellulose fibre matrix and the strong interaction between RGO and cellulose played an instrumental role in determining the performance of the composites. (**CA-JA4**)

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CA-JA4: Charu Agarwal, M. N. Singh, R. K. Sharma, Archna Sagdeo, Levente Csóka, "In Situ Green Synthesis and Functionalization of Reduced Graphene Oxide on Cellulose Fibers by *Cannabis sativa* L. Extract" *Materials Performance and Characterization* doi: 10.1520/MPC20180149, **2019**

CA-JA3: Charu Agarwal, Tamás Hofmann, Levente Csóka, "Ultrasonically-Extracted Phytocannabinoids from a Hungarian Chemovar of *Cannabis sativa* L.: Possible Entourage Effects?", *Food and Function* (submitted)

CA-JA2: Charu Agarwal, Levente Csóka, "Functionalization of Wood/Plant-Based Natural Cellulose Fibers with Nanomaterials: A Review", *TAPPI Journal* 17 (2), 92-111, **2018**

CA-JA1: Charu Agarwal, Katalin Máthé, Tamás Hofmann, Levente Csóka, "Ultrasound-Assisted Extraction of Cannabinoids from *Cannabis sativa* L. Optimized by Response Surface Methodology", *Journal of Food Science* 83 (3), 700-710, **2018**

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