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Phytochemical screening and antibacterial activity of green synthesis of nanoparticles of *Punica granatum*

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Abstract

The main objective of this work was to explore *Punica granatum* phytochemicaly and synthesize nanoparticles from its polar extract. The phytochemical screening test of ethanolic and aqueous extract of *P. granatum* indicated the presence of tannins, flavonoides, emodins, terpenoids, cardiac glycosides, coumarine, and soluble starch. The aqueous extract showed the presence of tannins, flavonoides, terpenoids, emodins, cardiac glycosides, caumarine, carbohydrates and soluble starch. The extracts were subjected to synthesize gold nanoparticles (Au-NPs) Synthesized Au-NPs were characterized by using Uv-visible, Fourier transform (FT-IR) spectroscopy and AFM (atomic force microscope) analysis. The active phytochemical present in extract are responsible for synthesizes gold nanoparticles (Au-NPs). In addition, synthesized Au-NPs and the methanolic extracts of *Punica granatum* were screened for their *in-vitro* antioxidant activity. Au-NPs showed excellent antioxidant potentials. It is concluded that *Punica granatum* extracts is an outstanding bioreducant for the rapid and green synthesis of Au-NPs, which in turn showed various antioxidant activity.

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Introduction

Medicinal plants are used by 80% of the world population for their basic health needs. The relationship between plants, humans and drugs derived from plants describe the history of mankind. Plants are the important source of natural drugs (Shahrajabian *et al*., 2019). The plants are assumed to contain compounds which have potential to be used in modern medicine for the treatment of diseases which are not curable. During the past decade, traditional systems of medicine have become a topic of global importance. Currently, many people in the developed countries have begun to turn to alternative or complementary therapies, including medicine herbs (Schippmann, 2002). Medicinal plants are a source for a wide variety of natural antioxidants and are used for the treatment of diseases throughout the world. Some of these properties are antimicrobial, anti-cancer, anti- diabetic, anti-atherosclerosis, immunomodulatory, and venreno-protection or hepato-protective effects (Rafieian 2013). Major reason of use of plants as medicines is that medicinal plants contain synergistic and/or side-effects neutralizing combinations.

A nanoparticle (or nanopowder or nanocluster or nanocrystal) is a microscopic particle with at least one dimension less than 100nm. Nanoparticle research is currently an area of intense scientific research, due to a wide variety of potential applications in biomedical, optical, and electronic fields. Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures (Hossain *et al*., 2020).

Nanoparticle Applications in Medicine:

The use of polymeric micelle nanoparticles to deliver drugs to tumors. The use of polymer coated iron oxide nanoarticles to break up clusters of bacteria, possibly allowing more effective treatment of chronic bacterial infections. The surface change of protein filled nanoparticles has been shown to affect the ability of the nanoparticle to stimulate immune responses (Seniya *et al*., 2011). Researchers are thinking that these nanoparticles may be used in inhalable vaccines.

Researchers at Rice University have demonstrated that cerium oxide nanoparticles act as an antioxident to remove oxygen free radicals that are present in a patient's bloodstream following a traumatic injury (Chidambara *et al*., 2002). The nanoparticles absorb the oxygen free radicals and then release the oxygen in a less dangerous state, freeing up the nanoparticle to absorb more free radicals. Researchers are developing ways to use carbon nanoparticles called nano-diamonds in medical applications (Daniel and Astruc, 2004).

Pomegranate is an erect deciduous spreading tree, 8 to 10 meters high; stem is woody and light yellow in color. Seeds with a fleshy aril which constitutes the edible part. It grows easily from seed, but is commonly propagated from 25–50 cm hardwood cuttings to avoid the genetic variation of seedlings. The pomegranate known as "anar" in Urdu) is a popular fruit in Pakistan (Sultana and Rahman, 2013). Pharmacological effects of pomegranate represent a long history. Recently, studies have shown that pomegranate has many impending effects including: bacteriocidal (Uddin *et al*., 2011), antifungal, antiviral, immune modulation, diuretic and moreover, it serves to decrease the adverse effects of cardiovascular diseases, diabetes (Lee *et al*., 2016), asthma, bronchitis, cough, bleeding disorders, fever, inflammation, acquired immune deficiency syndrome, dyspepsia, ulcers, sores, malaria, prostate cancer, atherosclerosis, hyper lipidemia, male infertility, alzheimer, obesity and infant brain ischemia (Rose *et al*., 2001). Therefore the aim of this study was to develop a green nanotechnology procedure to synthesized gold nanoparticles using *Punica granatum* methanolic extract and to observe the presences of active phytochemicals which were involved in nanoparticles synthesis.

Materials and methods

Collection of plants materials (for Methanolic crude extract)

The peel of *Punica grantum* was collected from Kotha (Topi) District Swabi. The peels were collected from Swabi (Kotha) District Swabi while *Pterospermuma cerifolium* stem and young shoots were collected from university of Peshawar campus, Peshawar in the month of March in 2012.

The authenticities of the plants were identified by taxonomist Ghulam Jelani, Department of Botany, University of Peshawar. The vouchers specimens were deposited in the Herbarium, Department of Botany, and University of Peshawar.

Drying and grinding of plants materials

The plants materials after collection is cleanly washed by water and then were shade dried under room temperature. About 5 kg of *Punica grantum (*peel), after drying were finely grinded by a local grinder machine to make powder. The powdered plants materials were further used for extraction of plants crude extract.

Extraction and Fractionation

Hot extraction technique was followed by using Soxhelt apparatus for the extraction of crude extract from powdered plants materials of *Punica grantum*, Methanol was used as extracting solvent. After extraction vacuum rotary was used to concentrate plants crude extracts and to evaporate methanol under low pressure at 40˚C. *D. salicifolia* give about 265g of crude extract while 250g was obtained from *P. acerifolium*. *C. didymus* and *C. lanatus* were extracted by cold extraction technique. At the end of the technique 110g of crude extract was obtained from *C. didymus* while *C. lanatus* gives 95g of crude extract. The powdered plants materials were dipped in the methanol for seven days in different extraction tanks at room temperature, after seven days the filtrate was concentrated by evaporating methanol using reduced pressure rotary evaporator at 40˚C. The residue was again dipped in methanol for more seven days for the purpose of complete extraction of crude extract from the powdered residue, followed by vacuum filtration. All of the plants crude extracts were stored after drying at 4˚C.

The fractionation was carried out using separating funnel for the purpose to separate different frictions from plants crude extracts according to polarity using organic solvents i.e. (*n*-hexane, chloroform, ethyl acetate and methanol) (Murad *et al.*, 2012) The plants crude extracts were suspended in water first then followed by adding organic solvent starting from nonpolar, so *n*-hexane was introduced to the separating funnel double as compared to water. The residual aqueous layer was again partitioned to different fraction using organic solvents like chloroform and ethyl acetate by following the same above technique to obtained chloroform and ethyl acetate fractions. Methanolic fraction was obtained by drying residual aqueous layer at 40˚C and then methanol was added to dry residue of crude extract. The soluble portion was rotary evaporated and separated as methanolic friction.

Photochemical screening

The extracted plants crudes and their sub fractions were analyzed for the presence of secondary metabolites using standard protocols (Rauf *et al.*, 2012).

Nanoparticles synthesis

Plants collections and extraction

Fresh plants materials of the selected medicinal plants i.e. *Debregeasia salicifolia*, *Coronopus didymus*, *Carthamus lanatus* and *Pterospermum acerifolium* were collected from different areas. About 100 g of fresh stem and shoots of *D. salicifolia* were collected from Dargai (Jabaan) District Malakand, 50 g of fresh plants of *C. didymus* and *C. lanatus* of each plant were collected from Takht Bhai (Fazal Abad) District Mardan and 100g fresh stem and shoots of *P. acerifolium* were collected from University of Peshawar campus. The fresh plants materials were washed cleanly by distilled water for the removal of dust and other particles. Then all of plants materials were finely cut into small pieces. Small pieces of fresh plants materials were put in a conical flask separately followed by the introduction of de-ionized water. The flasks were kept at 40˚C for 24 hours, after one day fractions were filtered and filtrates were concentrated and stored in the refrigerator at 4˚C.

Synthesis of nanoparticles (silver and gold nanoparticles)

Nanoparticles were synthesized by following simple procedure and without the addition of any reducing or capping agents. Stock and salt solutions were mixed together in a small round bottom flask in

various ratios i.e. 1:1, 1:2, 1:3, 1:4 and 1:5. The flask was connected to condenser to prevent water vapors to escape. The reaction mixture 1:1 was first heated at 30˚C for 30 minutes; color was noticed before heating and after some time. Then time was extended to 1 hour. The change in color showed the reduction process and the synthesis of nanoparticles. The above mentioned ratios were heated at 30˚C, 40˚C, 50˚C, 60˚C, 70˚C and 80˚C for 30, 45 and 60 minutes. UV-spectra were taken for each and every step to check and conform the synthesis of metallic nanoparticles.

UV- Visible analysis

The optical properties of Ag/Au NP's were determined by UV-Vis spectrophotometer. The uv was taken after different time interval at different ratio for different plants crude extracts. The nanoparticles which have the most suitable graph were taken for next characterization and biological activities. The range of uv-spectra were from 300 - 600nm in wavelength. The spectra was taken for the stock solution as well as when color change/ reduction due to the addition of metallic salt. The spectra's were compared to each other for the conformation of synthesis of nanoparticles.

FT-IR analysis

FT-IR instrument was used to find out the chemical composition of the plants crude extracts and synthesized nanoparticles. Both (plants crude extracts and synthesized nanoparticles) in dried powdered form were analyzed in the range from 4000 to 400 cm^{-1}. Potassium bromide (KBr) pellets method was followed for the analysis. Both the spectra were compared to each other for the conformation of nanoparticles synthesis.

Biological Screening (plants crude extract, sub-fractions and NPG's)

Plants crude extracts along with different sub-fractions and synthesized nanoparticles were subjected for different biological screening to evaluate and explore their therapeutic and medicinal importance. Mentioned activities have been done to evaluate biological activities: Antioxidant activity, antibacterial activity, antifungal activity.

Antioxidant activity

The antioxidant activity was done by DPPH radical scavenging activity according to standard protocol and previse literature reported (Philip *et al.*, 2011). The electron donation capabilities of the corresponding crude extracts, fractions, synthesized nanoparticles and standards were measured from the changing of the purple-colored methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). 3 ml of sample solution (plants crude extract, fractions and synthesized nanoparticles) was mixed with 1 ml of the 1mM solution of DPPH solution in methanol, various concentrations i.e. (10-100 µg/ml) for fractions, plants crude extract and synthesized nanoparticles have been prepared and control has only methanol and DPPH solution without sample. The mixed solutions were stand for 30 minutes in dark then absorbance was measured at exact 517nm. The decreasing of the DPPH solution absorbance shows an increase in the antioxidant activity. Antioxidant activity by DPPH as percent radical scavenging activities (%RSA) was calculated as follows.

$$\% \text{ DPPH} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100$$

Where, OD control is the absorbance of the blank sample, and OD sample is the absorbance of samples.

Antifungal activity

To find out the antifungal efficacy of plants crude extracts various sub-fractions and synthesized NP's were measured by following the previous reported work (Seebacher *et al.*, 2003).

Antibacterial activity

The plants crude extracts, different separated sub-fractions and synthesized NPG's were processed against various bacterial strains i.e. *Klebsiella pneumonia, Staphlococcus aureus, Staphlococcu sepidermidis, Bacillus sibtilis* to elevate antibacterial activity for the exploration of their therapeutic medicinal uses according to standard protocol (Uddin *et al.*, 2012).

Results and discussion

Phytochemical screening of P.granatum

The phytochemical screening test of ethanolic and aqueous extract of *P. granatum* is given in table 1.

The ethanolic extract indicated the presence of tannins, flavonoides, emodins, terpenoids, cardiac glycosides, caumarine, and soluble starch. The aqueous extract showed the presence of tannins, flavonoides, terpenoids, emodins, cardiac glycosides, caumarine, carbohydrates and soluble starch.

Table 1. Phytochemical assortment of *P. granatum* (peel) extract.

Chemical constituents	Ethanolic extract	Aqueous extract
Alkaloids	-	-
Tannins	+	+
Anthraquinones	-	-
Glycosides	-	-
Reducing Suger	-	+
Saponins	-	-
Flavonoids	+	+
Phlobatanins	-	-
Steroids	-	-
Terpenoids	+	+
Cardiac Glycoside	+	+
Caumarine	+	+
Emodines	+	+
Anthocyanin &Betacyanin	-	-
Carbohydrates	-	+
Monosaccharides	-	-
Reducing Sugar	-	-
Combined Reducing Sugar	+	-
Soluble Starch	+	+

Synthesis of nanoparticles

The biomolecules present in the plants induced the reduction of Au^{3+}, Ag^{+} ions from $H[AuCl_4]$ and $AgNO_3$ respectively, which resulted in the formation of nanoparticles. Green synthesis of NPs by bioreduction of aqueous metal ions was checked by UV-vis spectroscopy.

Change in the color easily indicates the formation of nanoparticles. Formation of Ag and Au nanoparticles at different ratios was done, efficient synthesis was observed by 1:1 AgNPs at 375nm for *C. didymus*, 1:2 AgNPs at 370nm for *H. hirsuta* and1:2 AuNPs at 435nm wavelength for *P. granatum* from fig. 5-8.

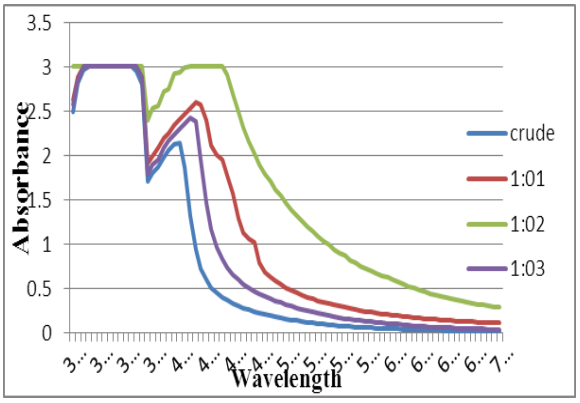

Fig. 5. UV spectrum of AuNPs at different ratios of *P. granatum* peel aqueous extract.

This spectra show that absorption takes place at a range from 400-500nm. Maximum the absorption maximum will be the size of nanoparticles.

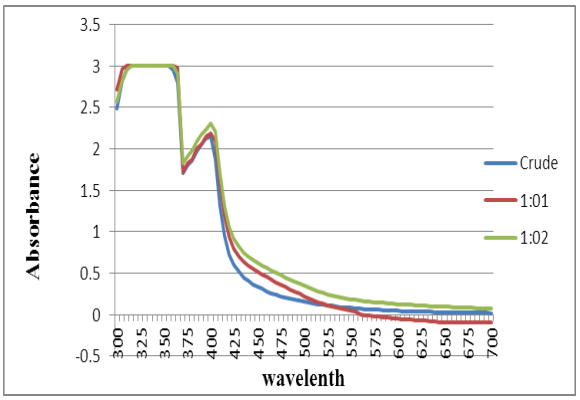

Fig. 6. UV spectrum of AuNP sat different ratios of *P. granatum* peel ethanolic extract.

It shows absorbance of the different ratios of the synthesized nanoparticles.

FT-IR Analysis of P. granatum

The crude extract of *P. granatum* showed a broad peak at 3200-3400cm^{-1} which show presence of alcoholic and phenolic groups, and sharp peak at 3000 cm^{-1} corresponds to C-H stretching frequency. A characteristic peak at 1000 cm^{-1} was observed which showed a presence of C-F stretching frequency. In case of Au nanoparticles the OH peak intensity further increased, while the peak at 3000 cm^{-1} completely disappeared which showed that O-H and C-H olefinic are involved in the formation of nanoparticles. Also a broad band at 500 cm^{-1} which shows presence of nanoparticles.

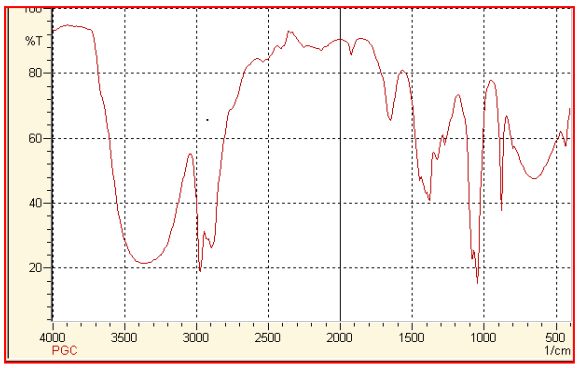

Fig. 7. FTIR spectra recorded for the crude extract of *P. granatum* peel.

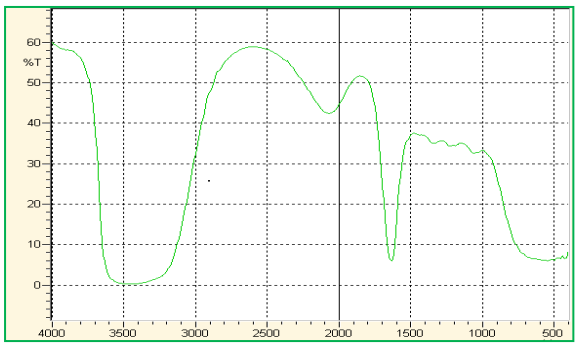

Fig. 8. FT-IR spectrum of AuNPs of *P. granatum* peel extract.

AFM imagining

To determine the size and shape of synthesized NPs AFM study was carried out. The images shows that NPs were spherical in shape. The fig. 19 represents AFM image of *P. granatum* AuNPs, it was concluded from the image that the NPs are from 4-16nm range. The fig. 20 represents AFM image of AgNPs of C. didymus which shows that NPs are in the range of 5-60nm. The fig. 21 shows AFM image of AgNPs of *H. hirsuta* which indicats that NPs are in the range of 1-6.5nm.

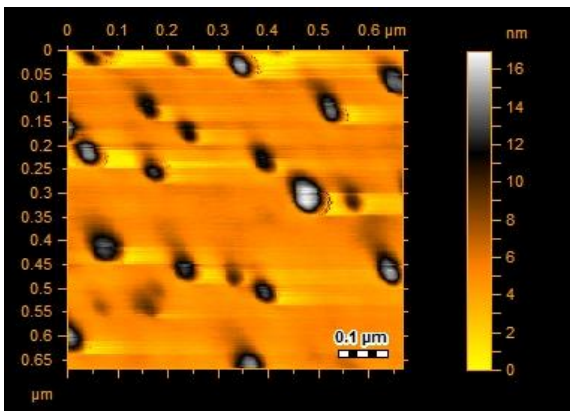

Fig. 9. AFM image of AuNPs of *P. granatum*.

Biological Activities

Antioxidant activity

Plant sourced food antioxidants which has potential to reduce different diseases like chronic and heart diseases. Most of the antioxidant compounds in a typical diet are derived from plant sources with wide variety of physical and chemical properties. DPPH is a stable free radical compound having ability to trap free radicals, and use to study the scavenging effect of plant with a characteristic absorption at 517nm.

Table 3, 4 and 5 represents the antioxidant profile of *P. granatum, C. didymus, H. hirsuta* and their synthesized nanoparticles. The maximum antioxidant potential at different concentration of crude extract and nanoparticles were given in the tables. It can be seen from the tables that methanolic extract of all plant along with NPs offered varying degrees of scavenging abilities. The results also showed that the scavenging activity was increased with increasing concentration for both extract and nanoparticles.

Table 4. Antioxidant activity of rude extract and AuNPs of *P.granatum*.

Crude Extract		Nanoparticles	
Conc µg/ml	%DPPH Activity	Conc µg/ml	%DPPH Activity
20	35.14	20	93.97
40	81.15	40	94.06
60	89.97	60	94.23
80	90.82	80	94.40
100	92.87	100	94.89
150	91.90	150	29.34

Conclusion

It is concluded that we have developed a green nanotechnology procedure to synthesized gold nanoparticles using *Punica granatum* methanolic extract. The extract was screen for presence of active phytochemical which are involved in nanoparticles synthesis. The characterization of nanoparticles was achieved by using UV, FT-IR, and AFM technique.

The crude extract and synthesized gold nanoparticles have promising antioxidant activity. This study has several advantages like a cost effective and compatibility for biomedicinal and pharmaceutical industry.

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